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TRAIL resistance through transcriptional control of *MCL-1*

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Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas at Austin

December 2009

Acknowledgements

I would like to thank my mentor, Dr. Shawn B. Bratton, for giving me this opportunity and for his constant guidance and encouragement. He has always been there for me and his passion and enthusiasm for science has allowed me to get more excited about science. Also, I would like to thank Dr. Mills, Dr. Richburg, Dr. Tang, and Dr. Wright for serving on my dissertation committee and providing helpful suggestions and comments.

I am grateful to all my lab members, Youngeun, Shankar, Madhavi, Srinivas, Indra, Daric, Ting-Chun, Crystal and Sophie for their support not only as lab members but also as friends.

I would like to thank my wife, Eunyoung, and my family for having been there for me all the time and for their love, encouragement, and help in completing my work successfully. Finally, I would take an opportunity to thank my daughter for making me happy all the time.

TRAIL resistance through transcriptional control of *MCL-1*

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The University of Texas at Austin, 2009

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Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a potentially useful anticancer agent with exquisite selectivity for cancer cells. Unfortunately, many cancers exhibit or acquire resistance to TRAIL. We report herein that TRAIL activates a TGF-ß-activated kinase 1→mitogen-activated protein kinase (MAPK) kinase 3 (MKK3)/MKK6→p38 pathway in prostate cancer cells that transcriptionally upregulates expression of the antiapoptotic BCL-2 family member MCL-1. TRAIL alone triggered robust formation of the "death-inducing signaling complex", activation of the initiator caspase-8, and truncation of the BH3-only protein BID (tBID). Nevertheless, simultaneous disruption of the p38 MAPK pathway was required to suppress MCL-1 expression, thereby allowing tBID to activate the proapoptotic BCL-2 family member BAK and stimulate mitochondrial outer membrane permeabilization (MOMP). Release of the inhibitor-of-apoptosis antagonist, Smac/DIABLO, from the intermembrane

space was sufficient to promote TRAIL-induced apoptosis, whereas release of cytochrome c and apoptosome function were dispensable. Even following MOMP, however, mitochondrial-generated reactive oxygen species activated a secondary signaling pathway, involving c-Jun N-terminal kinases, that likewise upregulated MCL-1 expression and partially rescued cells from death. Thus, stress kinases activated at distinct steps in the extrinsic pathway mediate TRAIL resistance through maintenance of MCL-1 expression.

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List of Abbreviations

AIF	apoptosis-inducing factor
APAF-1	apoptotic protease-activating factor-1
ASK1	apoptosis signal-regulating kinase 1
ATF1	activating transcription factor 1
ATP	adenosine triphosphate
BH	Bcl-2 homology
BIR	baculovirus IAP repeat
CARD	caspase activation and recruitment domain
caspases	cysteinyl aspartate-specific proteases
CDK	cyclin-dependent kinase
c-FLIP	Cellular-FLICE-like inhibitory protein
cIAP1/2	cellular inhibitor of apoptosis-1/2
CRD	cysteine-rich domain
CREB	cAMP response element-binding protein
DD	death domain
DcR	Decoy receptor
DED	death effector domain
DISC	death inducing signaling complex
ER	endoplasmic reticulum

FADD	Fas-Associated protein with Death Domain
GSK	Glycogen synthase kinase
HIV-1	human immunodeficiency virus-1
HSP27	heat shock protein 27
H_2O_2	hydrogen peroxide
IAP	inhibitor of apoptosis
IBM	IAP binding motif
ICAD	inhibitor of caspase-activated deoxyribonuclease
IFN	interferon
IKK	IkB kinase
IL	interleukin
ILP2	IAP-like protein 2
IMM	inner mitochondrial membrane
IMS	intermembrane space
JAK	Janus kinase
JIP	JNK interacting protein
JNK	c-Jun N-terminal kinase
LC3	Microtubule associated protein 1 light chain 3
LSP1	lymphocyte-specific protein 1
MAPK	Mitogen-activated protein kinase
MAPKAPK2	MAPK-activated protein kinase 2

MCL-1	myeloid cell leukemia-1
MKK	MAPK kinase
MKP	MAPK phosphatase
MKKK(M3K)	MAPK kinase kinase
MLK	mixed-lineage protein kinase
MOMP	mitochondrial outer membrane permeabilization
Mule	MCL-1 ubiquitin ligase E3
NAIP	neuronal apoptosis inhibitory protein
NBD	nucleotide binding domain
NEMO	NF-κB essential modulator
NF-κB	nuclear factor-κB
NIK	NF-κB Inducing kinase
NK	natural-killer
$OH^{\cdot}$	hydroxyl radical
OMM	outer mitochondrial membrane
OPG	osteoprotegerin
$O_2^{\cdot -}$	superoxide anion
PARP	poly (ADP-ribose) polymerase
PE	phosphatidylethanolamine
PP2Calpha	protein phosphatase type 2Calpha
PTPase	protein tyrosine phosphatase

RIP	receptor interacting protein
ROS	reactive oxygen species
Smac/DIABLO	second mitochondria-derived activator of caspases / direct IAP binding protein with low pI
SRF	serum response factor
TAB	TAK-binding protein
TAK1	transforming growth factor beta-activated kinase 1
tBid	truncated Bid
TCR	T cell antigen receptor
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
TRADD	TNFR1-Associated Death Domain
TRAF	TNF receptor-associated factor
TRAIL	TNF-related apoptosis-inducing ligand
XIAP	X-linked IAP
zVAD-fmk	zValine-Alanine-Aspartate-fluoromethyl ketone
$\Delta\psi_m$	mitochondrial membrane potential

Chapter 1. Introduction

1.1 Apoptosis

Apoptosis is a programmed form of cell death that plays an important role during development and helps maintain tissue homeostasis by ridding the body of unwanted, damaged, or infected cells (Kerr et al., 1972). In multicellular organisms, the total number of cells is a balance between the cell-generating effects of mitosis and cell death that is induced through apoptosis. Therefore, a disruption of this delicate balance is associated with many diseases and disorders from cancer to neurodegeneration (Fadeel et al., 1999b).

Several types of cell death have been described including, apoptosis, autophagy, and necrosis (Kroemer et al., 2005). Cells undergoing apoptosis show typical, well-defined morphological changes, including plasma membrane blebbing, chromatin condensation with margination of chromatin to the nuclear membrane, karyorhexis (nuclear fragmentation), cytoskeletal collapse, and ultimately intact membrane-wrapped "apoptotic bodies" that are engulfed by adjacent phagocytes (Kerr et al., 1972). Apoptosis has been characterized by several biochemical criteria, including caspase activation, phosphatidylserine (PS) exposure on the outer leaflet of the plasma membrane (Fadok et al., 1992), changes in mitochondrial membrane permeability (Kroemer and Reed, 2000), release of proteins from the mitochondrial intermembrane space (Van Loo et al.,

2002), and caspase-dependent activation and nuclear translocation of DNase that induces internucleosomal DNA cleavage (Enari et al., 1998). Identification of these morphological and biochemical markers of apoptosis makes it possible to distinguish it from other forms of cell death. Cells undergoing autophagy are characterized by the presence of double membrane autophagic vacuoles. Autophagy is primarily a survival mechanism that is activated in cells subjected to nutrient or obligate growth factor deprivation. When cellular stress continues, cell death may continue by autophagy alone, or more often becomes associated with features of apoptotic or necrotic cell death. Specific biochemical markers have been determined for autophagy, such as conjugation of light chain 3 (LC3) to the phosphatidylethanolamine (PE) on autophagosomal membranes, which can be detected as a band shift by western blotting (Kabeya et al., 2000). In contrast, necrosis is characterized by rapid cytoplasmic swelling. It culminates in rupture of the plasma membrane and breakdown of organelles (Majno and Joris, 1995). Necrosis has long been described as a consequence of extreme physicochemical stress such as heat, osmotic shock, mechanical stress, freeze-thawing, or exposure to reactive oxygen species (ROS). Under these conditions, necrotic cells undergo cytosolic and mitochondrial swelling, resulting in loss of membrane integrity and cell lysis, and this death occurs quickly due to the direct effect of the stress on the cell. Therefore, this cell death process has been described as accidental and uncontrolled.

As already noted, apoptosis is implicated in many diseases because it is critical for various aspects of development, including the removal of structures with transient functions, tissue sculpting, and morphogenesis. Thus, proper regulation of apoptosis is important to maintain the health of living organisms and deregulation can contribute to the pathogenesis of various diseases (Thompson, 1995). Apoptosis has been shown to play an important role in cancer, human immunodeficiency virus-1 (HIV-1) infection, autoimmune diseases, and neurodegenerative disorders such as Alzheimer's, Huntington's, and Parkinson's disease. In each case, apoptosis is either abnormally inhibited (as in cancer) or is induced as in the case of HIV-1 infection. Among the diseases associated with inhibition of apoptosis, cancer is the second leading cause of mortality in the world. The successful treatment of cancer therefore is one of the greatest challenges of modern medicine. Although many scientists have made considerable strides in understanding how tumor cells survive and die over the past 20 years, and conventional treatments such as radiotherapy and chemotherapy continue to benefit patients with cancer, significant advances in survival are not expected with these therapies alone. Thankfully, by understanding the molecular mechanisms of apoptosis more rational approaches to cancer treatment are in development. One promising approach is the design of TRAIL receptor agonist and considerable progress has been made in this area. More

information about TRAIL and its roles in apoptosis will be covered in section 1.2.1.2.

1.2 Apoptotic pathways

The execution of apoptosis is made possible by a group of cysteinyl aspartate-specific proteases known as caspases. Caspases are synthesized in the cytosol as 30-50 kD single-chain zymogens, and in most cases are activated following intramolecular cleavage at specific aspartate residues (Cohen, 1997). Two well-characterized signaling pathways that activate caspase cascades, leading to activation of apoptosis, are the death receptor (extrinsic) and the mitochondrial (intrinsic) pathways. These two pathways are initiated in response to different stimuli, which ultimately converge to activate the effector caspases leading to cellular demise in a similar fashion.

Diverse stressors activate the intrinsic pathway, which results in release of cytochrome c from the mitochondrial intermembrane space into the cytoplasm (Li et al., 1997), followed by dATP/ATP-dependent formation of a multimeric apoptotic protease-activating factor-1 (Apaf-1) apoptosome complex (Acehan et al., 2002; Cain et al., 1999) . This complex recruits and activates caspase-9 and other effector caspases, that can further cleave numerous substrates like ICAD, PARP, lamins, cytokeratins, etc. to induce apoptosis (Bratton and Cohen, 2001; Bratton et al., 2000; Bratton et al., 2001). In addition to cytochrome c, other

apoptosis-promoting factors are also released from several sub-cellular compartments to induce apoptosis. More information about the intrinsic pathway will be covered in section 1.2.1.

Similarly, the extrinsic pathway is generally activated in response to external signals in the form of death ligands (e.g TNF, TRAIL, and CD95L). These are potent cytokines, produced by many cell types, including macrophages, monocytes, lymphocytes, keratinocytes, and fibroblasts in response to inflammation, infection, injury, and other challenges. When these ligands interact with their corresponding receptors, a membrane-bound, caspase activating multiprotein assembly called the 'death-inducing signaling complex' (DISC) is formed (Scaffidi et al., 1999). Some components in the DISC facilitate the binding and activation of procaspase-8 and as a result, apoptosis ensues. However, the DISC also induces the expression of inflammatory antiapoptotic genes that counteract the cell death response in a cell-type and stimulus-specific manner.

There are several mechanisms through which the assembly of the DISC or the apoptosome complex can be prevented, but once they are formed and begin activating caspases, the inhibitor of apoptosis (IAP) protein become the main inhibitor of cell death. In normal cells that have not received an apoptotic stimulus, most caspases remain inactive. Even if some caspases are aberrantly activated, their proteolytic activities or presence in the cell can be fully regulated

by the evolutionarily-conserved IAPs (Choi et al., 2009; Deveraux et al., 1998; Eckelman and Salvesen, 2006). Several distinct mammalian IAPs, including X-linked IAP (XIAP), cellular IAP 1 (cIAP1), cIAP2, neuronal apoptosis inhibitory protein (NAIP), survivin, ML-IAP/Livin, BRUCE/Apollon, and IAP-like protein 2 (ILP2) have been identified, and they all exhibit antiapoptotic activity in different contexts, though only XAIP directly inhibits caspase activity. Each of the IAPs contains one or more tandem repeats of ~70 amino acids, forming the Baculoviral IAP Repeat domains, which in the case of XAIP, directly interact with and inhibit the enzymatic activity of certain mature caspases, namely caspase-3, -7, and -9 through its linker-BIR2 and BIR3 domains (Sun et al., 2000; Takahashi et al., 1998). In some cases, IAPs also contain a caspase-recruitment domain (CARD), a UBA domain, and/or a carboxy-terminal RING zinc-finger domain, conferring an ubiquitin E3 ligase activity (Deveraux and Reed, 1999).

Apoptotic signals require that the IAP-mediated inhibitory effect on caspases be removed; a process performed by a mitochondrial protein named Smac (second mitochondria-derived activator of caspases) or DIABLO (direct IAP binding protein with low pI) (Du et al., 2000; Verhagen et al., 2000). Upon apoptotic stimuli, Smac/DIABLO is released from the intermembrane space of mitochondria into the cytosol, together with cytochrome c. Whereas cytochrome c directly activates Apaf-1 to facilitate formation of the apoptosome, Smac/DIABLO interacts with multiple IAPs and relieves their inhibitory effects

on both the initiator caspase-9 and the effector caspase-3 and -7 (Shi, 2002). In addition to Smac, other pro-apoptotic factors like Omi/ HtrA2 also interact with IAPs to induce apoptosis (Martins et al., 2002; Suzuki et al., 2001; Verhagen et al., 2002).

1.2.1 The extrinsic death receptor pathway

The extrinsic pathway of apoptosis is brought about, when death ligands bind and activate their corresponding death receptors to recruit and activate caspases. Most of these death ligands belong to the TNF family and are type II transmembrane proteins (Smith et al., 1994). Specific metalloproteases of the ADAM family, however, can cleave these ligands to generate soluble forms (Idriss and Naismith, 2000).

1.2.1.1 TNF and TNFR1 signaling

Tumor necrosis factor (TNF), a major mediator of apoptosis as well as inflammation and immunity, has been implicated in the pathogenesis of a wide spectrum of human diseases, including sepsis, diabetes, cancer, osteoporosis, multiple sclerosis, rheumatoid arthritis, and inflammatory bowel diseases (Chen and Goeddel, 2002). TNF signals through two distinct cell surface receptors – TNF receptor1 (TNFR1) and TNFR2. Most cell types constitutively express TNFR1 while TNFR2 expression is highly restricted to cells of the immune

system (Smith et al., 1994; Tartaglia and Goeddel, 1992). The interaction of the trimeric TNF ligand with its receptors results in formation of the DISC at the cell membrane, (Chinnaiyan et al., 1996; Hsu et al., 1996a; Hsu et al., 1996b; Hsu et al., 1995) which triggers a series of intracellular events that ultimately result in the activation of two major transcription factors, nuclear factor κB (NF-κB) and AP-1 (Brenner et al., 1989; Osborn et al., 1989). These transcription factors are responsible for the inducible expression of genes important for diverse biological processes, including cell growth and death, development, oncogenesis, immunity, inflammation, and other stress responses (Chen and Goeddel, 2002). TNF can simultaneously activate another signaling pathway with a strikingly different outcome. This involves the recruitment of Fas-associated death domain (FADD) and caspase-8 to the TNFR1 complex, where caspase-8 becomes activated, presumably by dimerization and self-cleavage, and initiates a protease cascade that leads to apoptosis (Wallach et al., 1999) (Figure 1.1).

Membrane-bound TNF (mTNF) is present as a trimer and is cleaved specifically by TNFα-converting enzyme (TACE) to generate its soluble form (sTNF) (Black et al., 1997; Tang et al., 1996). Moreover, mTNF binds and activates TNFR1 and TNFR2, while sTNF interacts with both receptors but reportedly activates only TNFR1 (Grell et al., 1998). The extracellular domains of TNF receptors have cysteine-rich domains (CRD), which are pseudorepeats containing six cysteine residues forming three disulfide linkages (Bodmer et al.,

2002). The cytoplasmic tail of the receptor contains a stretch of 60-70 amino acids making up the death domain (DD) (Ashkenazi and Dixit, 1998), which plays the primary role in forming the DISC complex, mediated through protein-protein interactions. The first protein to interact with the DD of TNFR1 is TNFR1-associated death domain protein (TRADD), which acts as a platform to bind three other proteins including TNF receptor associated factor2 (TRAF2), receptor-interacting protein 1 (RIP1) and FADD (Chinnaiyan et al., 1996; Hsu et al., 1996a; Hsu et al., 1996b). The TRADD-TRAF2-RIP complex is known to be essential for NF-κB activation, whereas the interaction between TRADD and FADD allows the recruitment and activation of caspase-8 and facilitates the apoptotic wing of the response (Hsu et al., 1995).

TRAF2 like other TRAF proteins has a C-terminal TRAF domain and an N-terminal RING Zn finger domain (Takeuchi et al., 1996). The N-terminus is important for c-jun N-terminal kinase (JNK) and p38 MAPK activation, *via* interactions with several MAPK kinase kinases (MKKKs) like transforming growth factor-ß-activated protein 1 (TAK1) and apoptosis signal-regulating kinase 1 (ASK1) (Carpentier et al., 1998; Ichijo et al., 1997b; Rothe et al., 1995b), and the C-terminal domain, called TRAF domain, is further subdivided into a more divergent N-proximal (TRAF-N) and a highly conserved C-proximal (TRAF-C) subdomain. The TRAF domain is responsible for trimerization and interaction with adapter molecules in TNFR1 signaling. The TRAF-N domain

contains a coiled-coil domain that forms a single alpha-helix, and the TRAF-C domain forms a novel eight-stranded anti-parallel beta-sandwich (Park et al., 1999). Both domains are necessary to form a trimer in the shape of a mushroom, with the TRAF-C domain as the cap, and the TRAF-N domain as the stalk (McWhirter et al., 1999). Furthermore, interaction of TRAF2 with TRADD requires a C-terminus of the TRAF-C domain, whereas interaction with RIP1 occurs *via* an N-terminus of the TRAF-C domain (Takeuchi et al., 1996). In addition to activating JNK and p38 MAPK, TRAF2 is critical for recruiting IkB kinase (IKK) complex to TNFR1 and activating the IKK complex, composed of three subunits including IKKα, IKKß, and IKKγ (NEMO) (Devin et al., 2000). The IKK complex then activates NF-κB by promoting phosphorylation-dependent degradation of inhibitor of NF-κB (IkB). Although TRAF2-/- fibroblasts exhibit a total loss of TNF-induced JNK activation, there is just a decrease in NF-κB activation (Yeh et al., 1997), and TNF-induced NF-κB activation is not significantly inhibited in TRAF5-/- fibroblasts (Nakano et al., 1999). However, TRAF2-/- and TRAF5-/- fibroblasts show a significant inhibition of NF-κB activation (Tada et al., 2001), suggesting that TRAF2 and TRAF5 play a redundant role in the TNF-induced NF-κB activation. Cellular IAPs (cIAPs) are recruited in the TNFR1 DISC assembly through interaction with TRAF2 *via* their BIR domain (Rothe et al., 1995a). Although cIAPs might appear to participate in NF-κB signaling, the precise mechanism underlying this activity is not known in

the TNFR1 signaling. However, recent study indicates that TNFα-induced NF-κB activation is significantly inhibited in the absence of both cIAP1 and cIAP2 (Varfolomeev et al., 2008). In addition to modulating TNFα-induced NF-κB activation, cIAPs have been shown to suppress caspase-8 activation in TNFα-induced apoptosis (Wang et al., 1998).

TRADD in addition to binding with TRAF2 also binds with RIP1 that has been found to be a key effector in NF-κB activation by TNFR1 (Hsu et al., 1996a). Furthermore, a C-terminal caspase-8-dependent cleavage of RIP1 blocks NF-κB activation, and promotes cell death (Lin et al., 1999). In addition, RIP1 lacking an intermediate domain drastically reduced TNF-mediated p38 activation (Yuasa et al., 1998). In direct contrast to what was observed with TRAF2 -/- cells, cells devoid of RIP1 do not mount any NF-κB response, but still activate JNK (Kelliher et al., 1998). The NF-κB activating property of RIP1 is independent of its kinase activity (Ting et al., 1996) and for this reason, RIP1 is considered a critical adaptor protein, helping in the recruitment of IKKs to the activated TNFR1 complex *via* interaction with NEMO (NF-κB essential modulator or IKKγ) (Zhang et al., 2000). Degradation of RIP1 following ubiquitination is achieved by A20, another factor recruited to the DISC that serves as a check point for RIP1-mediated NF-κB activity and apoptosis (Lee et al., 2000; Wertz et al., 2004).

The recruitment of FADD and caspase-8 to the TNFR1 DISC has been challenged by the finding that neither of these components were found in the

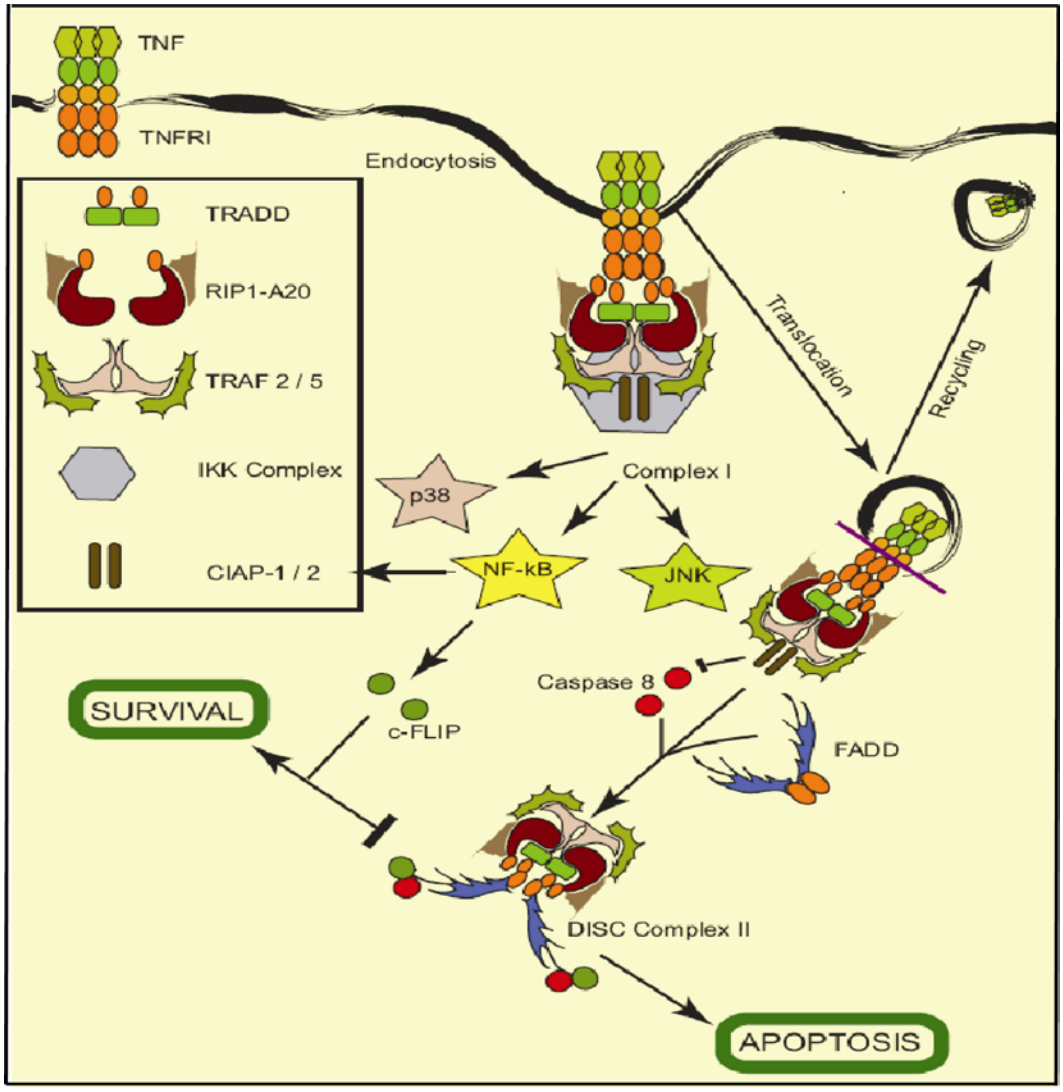


Figure 1.1 TNFR1 signaling

The initial step in TNFR1 signaling - the binding of TNF to TNF-R1 results in the recruitment of TRADD, which recruits additional adaptor proteins, like RIP and TRAF2 to the cytoplasmic death domains of TNFR1. TRAF2 recruits cIAPs1 and 2 and other TRAFs, thus forming complex I that can activate the IKK/ NF-κB and other MAPK pathways. Subsequently, this complex undergoes biochemical modifications and dissociates from TNFR1, and interacts with FADD and caspase 8, forming the DISC (complex II) in the cytosol. Binding of caspase-8 causes its activation and results in apoptosis.

TNFR1 receptor complex isolated from cells, following stimulation (Harper et al., 2003). It has been shown that upon stimulation with TNF, the trimeric receptor assembles all its cytoplasmic components in lipid rafts. This initial complex, called complex I, containing TNFR1, TRADD, TRAF2, RIP, and possibly other proteins, triggers the activation of NF-κB (Micheau and Tschopp, 2003). However, formation of complex I is transient and a large portion of the cytoplasmic protein components that constitute the DISC dissociate within an hour of stimulation. After dissociation, the DD of TRADD is available for interacting with the DD of FADD and as a result, caspase-8 gets recruited and activated (complex II) in the cytosol or in the endocytic vesicles (Micheau and Tschopp, 2003; Schneider-Brachert et al., 2004) (Figure 1.1). Afterwards, activated caspase-8 can either activate other downstream effector caspases, like caspases-3 and -7 (as in type I cells), or if activated only in very small quantities owing to inefficient DISC formation, caspase-8 can truncate BID, to form tBID, leading to release of cytochrome c which in turn activates the intrinsic pathway (as in type II cells) (Kuwana et al., 1998; Luo et al., 1998). Activation of caspase-8 can be inhibited through recruitment of FLIP to the DISC, which competes with procaspase-8 for binding to FADD, thereby blocking cell death (Irmler et al., 1997; Micheau et al., 2001).

1.2.1.2 TRAIL and TRAIL receptor signaling

TRAIL was discovered independently by two groups as a proapoptotic ligand that belongs to the *TNF* gene family (Pitti et al., 1996; Wiley et al., 1995). This ligand was called TRAIL or Apo-2L, respectively, because of its high homology to other members of the TNF family including Fas/Apo-1L (Pitti et al., 1996; Wiley et al., 1995). The *TRAIL* gene is located on chromosome 3 and spans approximately 20 kb, which is composed of five exons and four introns. TRAIL is a type II transmembrane protein and consists of 281 and 291 amino acids in the human and murine, respectively, which share 65 % identity. TRAIL has a short cytoplasmic N-terminus that is not conserved across the other TNF family members, whereas its long C-terminal extracellular domain shows significant conservation. The percent identity to the more related members of the TNF family, FasL and TNFα, is 28 and 22%, respectively (Wiley et al., 1995). TRAIL is mostly expressed on the surface of natural-killer (NK) cells and cytotoxic T cells and seems to play a role in the killing of virus-infected or malignant cells by these immune effector cells (Smyth et al., 2003). TRAIL is different in structure from the other prototypical ligands such as TNFα and FasL in structure. After cleavage of the extracellular C-terminal portion of TRAIL from the cell surface, TRAIL is released by the cells as a soluble form (Mariani et al., 1997). The soluble form of TRAIL comprising amino acids 114 to 281 is biologically active (Marsters et al., 1996; Pitti et al., 1996). Similar to the other TNF family members, TRAIL is a homotrimeric molecule. However, TRAIL is the only protein of the TNF family

that contains an essential cysteine residue, cys230, which allows the interaction among three molecules of TRAIL. The central zinc ion bound by the cysteines in the trimic ligand is essential for trimerization, solubility, stability, and biological activity (Bodmer et al., 2000b; Hymowitz et al., 1999; Mongkolsapaya et al., 1999).

1.2.1.2.1 TRAIL receptors

Five human TRAIL receptors have been identified. The first receptor, TRAIL-R1 (DR4/APO-2), was indentified in 1997 (Pan et al., 1997b). Another receptor, TRAIL-R2 (DR5/KILLER/TRICK2) was described in the same year (Pan et al., 1997a; Screaton et al., 1997; Sheridan et al., 1997; Walczak et al., 1997; Wu et al., 1997). These receptors can signal for cell death and share a sequence homology of 58 percent, but whether they have distinct functions remains unclear. The cytoplasmic domains of these receptors share significant homology with the DDs found in FasL and TNFR1. The cysteine-rich domains in the extracellular part are another common feature of these receptors. In addition to the apoptosis-inducing receptors, TRAIL-R1 and R2, two receptors have been found which fail to transmit the apoptotic signal, namely TRAIL-R3 (DCR1/TRID) (Degli-Esposti et al., 1997b; Pan et al., 1997b) and TRAIL-R4 (DCR2) (Degli-Esposti et al., 1997a), also called decoy receptors. Both receptors share high homology with the extracellular domain of the apoptosis-inducing

receptors. However, TRAIL-R3 lacks the complete intracellular DD that is essential for signaling apoptosis, and TRAIL-R4 has a cytosolic portion which contains only a truncated DD. Thus, neither receptor is able to induce apoptosis. Instead of induing apoptosis, they inhibit TRAIL-R1 and TRAIL-R2-mediated apoptosis. Overexpression experiments have shown that the decoy receptors compete with TRAIL-R1 and/or TRAIL-R2 for binding to TRAIL (Degli-Esposti et al., 1997a; Degli-Esposti et al., 1997b). Finally, osteoprotegerin (OPG) has been reported as a fifth receptor for TRAIL (Emery et al., 1998). OPG acts mainly as a regulator in the development and activation of osteoclasts during bone remodeling. However, this is not due to the interaction of OPG with TRAIL. OPG has also been proposed to function as a decoy receptor by binding TRAIL, and thus blocking apoptosis (Pritzker et al., 2004). Thus, the role of OPG as a TRAIL receptor is less well characterized compared with the other receptors.

1.2.1.2.2 Physiological roles of TRAIL

TRAIL is a cytokine that is expressed by effector lymphocytes and plays a role in shaping and regulating the immune system. The expression level of TRAIL is extremely low in freshly isolated lymphocytes (Takeda et al., 2001a). Furthermore, only a small set of murine NK cells express detectable TRAIL, and it appears most likely that the expression of TRAIL on liver NK cells is regulated by secretion of interferon (IFN)-γ from NK cells (Takeda et al., 2001b).

Stimulation of dendritic cells with IFN-ß has been shown to increase the expression of TRAIL by enhancing the cytotoxicity of dendritic cells on tumor cells (Liu et al., 2001b). Thus, TRAIL plays an important role in regulating the innate immune response involving NK and dendritic cells. TRAIL also contributes to the host immunosurveillance against metastasis, an effect apparently restricted to liver NK cells. Both neutralizing anti-mTRAIL mAB and TRAIL knockout mice support a direct role for TRAIL expressing NK cells in the suppression of tumor metastasis, (Cretney et al., 2002; Takeda et al., 2001a). In addition to the physiological role of TRAIL in tumor immunesurveillance, TRAIL is also implicated in regulating autoimmunity. Several studies have reported increases in the serum levels of soluble TRAIL in patients with systemic lupus erythrmatosus (Lub-de Hooge et al., 2005), and TRAIL may serve as a potential marker in determining the sensitivity of multiple sclerosis patients to INF treatment (Wandinger et al., 2003).

1.2.1.2.3 TRAIL receptor signaling

TRAIL-induced apoptotic signaling is reportedly simpler than TNFR1 signaling, but TRAIL triggers apoptosis through formation of DISC similar to TNF. The interaction of TRAIL with its two death receptors, DR4 and DR5, is the initial step, which in turn leads to recruitment of FADD through its DD and caspase-8 through its N-terminal death effector domain (DED). Formation of the

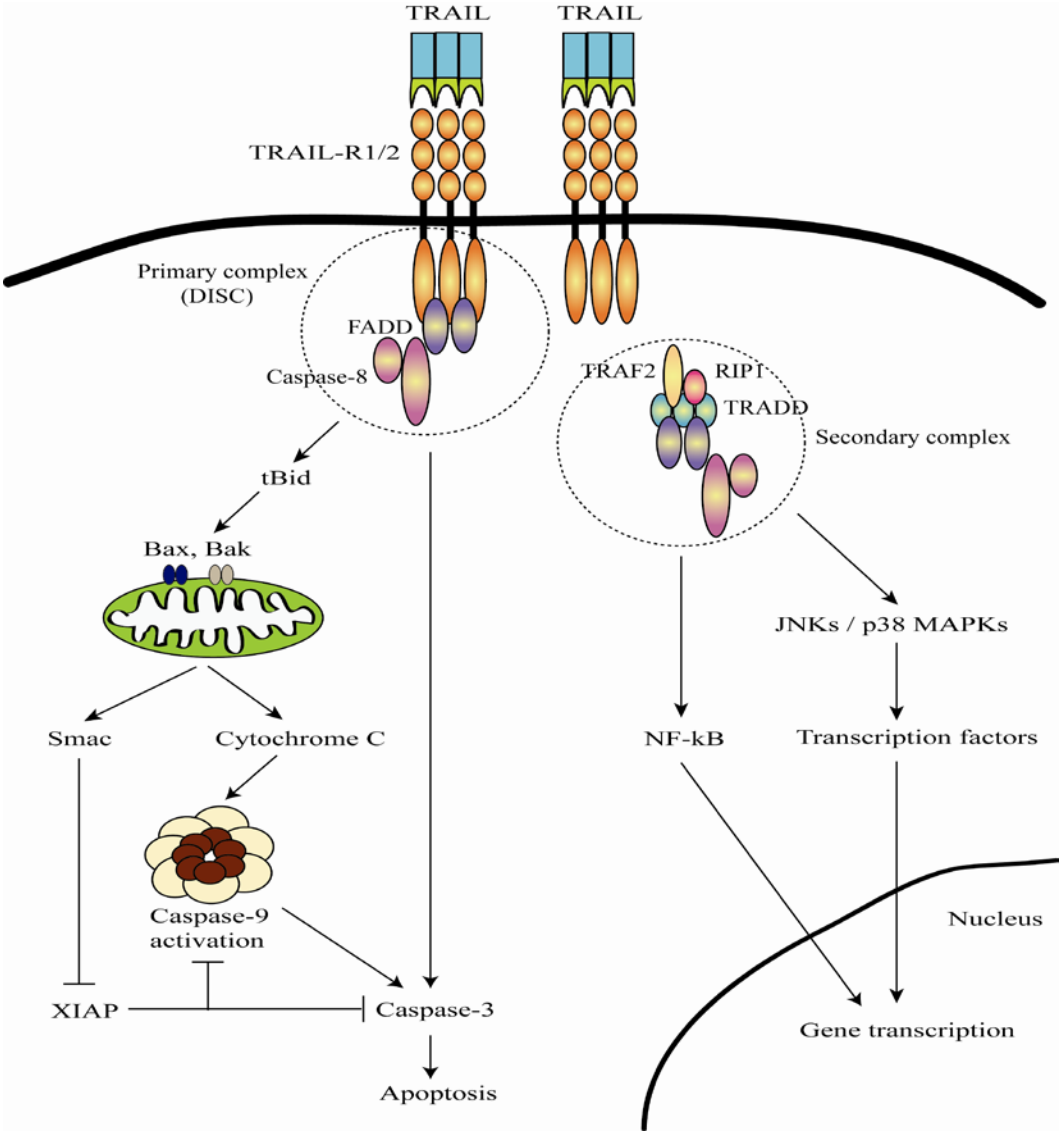


Figure 1.2 TRAIL receptor signaling

TRAIL induces apoptosis and pro-survival signaling. Activation of TRAIL-R1 and/or -R2 induces trimerization and formation of a primary DISC complex in lipid rafts, leading to apoptosis signaling. DISC-induced caspase-8 activation either directly activates the effector caspase-3 and triggers apoptosis or does so indirectly via Bid cleavage and activation of the intrinsic pathway. A secondary signaling complex is thought to be formed outside lipid raft structures, leading to activation of the transcription factor NF-κB and MAPKs and increased expression of antiapoptotic proteins.

TRAIL DISC promotes the activation of procaspase-8 by inducing dimerization within the DISC and this dimerization leads to its catalytic activation. Caspase-10 is also recruited to FADD through its DEDs and is cleaved with similar kinetics as caspase-8 (Kischkel et al., 2001). However, the importance of caspase-10 for the DISC is unclear and controversial. Several previous studies have failed to identify caspase-10 in the DISC or detect caspase-10 activation by engagement of FasL or TRAIL receptors (Bodmer et al., 2000a; Juo et al., 1998; Kischkel et al., 2000; Sprick et al., 2000). Once activated, caspase-8 activates the downstream effector caspase-3, which dismantles the cells. Like TNFR1 signaling, caspase-8 also cleaves Bid and activates the intrinsic pathway (Figure 1.2). Unlike FasL and TNF, however, there is currently no evidence to suggest that internalization of the TRAIL DISC is required for cell death (Kohlhaas et al., 2007). In addition to inducing apoptosis, TRAIL receptor stimulation also leads to activation of various signaling pathways, including activation of the IKK complex, MAPKs, and JNKs (Devin et al., 2000; Harper et al., 2003; Varfolomeev et al., 2005) (Figure 1.2). Although not required for apoptosis, TRAIL receptors reportedly undergo internalization and caspase-8-dependent formation of a secondary signaling complex (Kohlhaas et al., 2007; Varfolomeev et al., 2005). This intracellular complex contains RIP and TRAF2, which then signal for the activation of IKK and the stress kinases, p38 MAPKs and JNKs, through ill-defined mechanisms (Varfolomeev et al., 2005). Robust activation of these pathways also occurs

following treatment with TNF, and in particular, activation of the transcription factor NF-κB by IKK suppresses TNF-induced apoptosis in most cell types through upregulation of various antiapoptotic genes (Kelliher et al., 1998; Micheau and Tschopp, 2003; Song et al., 1997; Tobiume et al., 2001; Yuasa et al., 1998). By comparison, TRAIL activates these pathways to a lesser extent and their importance in regulating TRAIL-induced apoptosis remains unclear (Varfolomeev et al., 2005).

1.2.2 The intrinsic mitochondrial pathway

The intrinsic pathway is activated in response to developmental cues, growth factor withdrawal, DNA damage, cytoskeletal damage, oxidative stress, lysosomal injury, endoplasmic reticulum (ER) stress, etc. Mitochondria are involved in the production of ATP *via* oxidative phosphorylation, and they possess an inner mitochondrial membrane (IMM), which surrounds the matrix, and an outer mitochondrial membrane (OMM), which surrounds and separates the inter membrane space (IMS) from the cytoplasm (Fadeel et al., 1999a) (Figure 1.3). The IMM forms cristae and contains molecules, such as cytochrome c, which functions as an electron carrier, transferring electrons from complex III to complex IV during oxidative phosphorylation. Mitochondria maintains an inner membrane potential ($\Delta\psi_M$) by pumping H^+ from the matrix into the IMS, and this

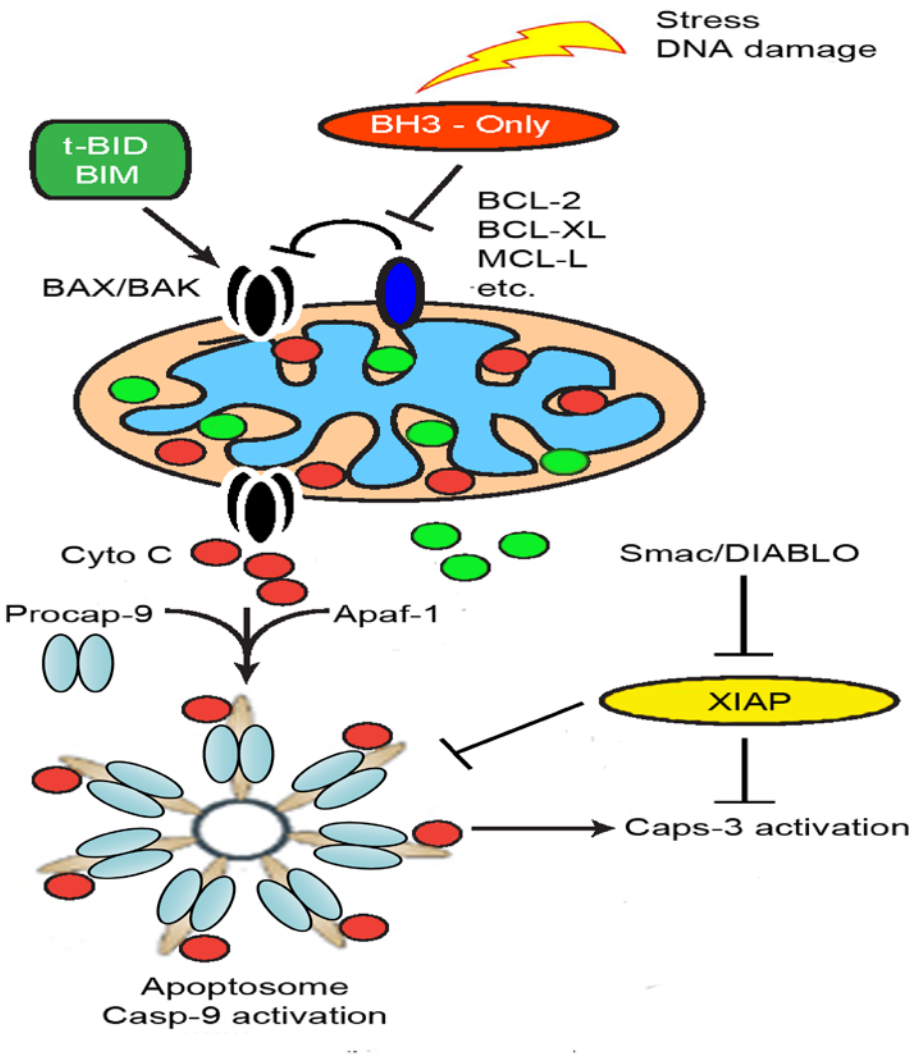


Figure 1.3 The intrinsic mitochondrial pathway

The intrinsic pathway is regulated by proapoptotic and antiapoptotic BCL-2 family members. When cells are damaged by death stimuli, BAX and/or BAK undergo a conformational change leading to their activation, which in turn results in release of several proapoptotic factors into the cytosol, including cytochrome c and Smac/DIABLO. Cytochrome *c* then triggers formation of the apaf-1·caspase-9 apoptosome, which activates caspase-3. On the other hand, Smac/DIABLO antagonizes XIAP, which relieves the inhibition of caspases including caspases-3,-9, and -7 by XIAP. The figure is modified from Chapter 2. Alterations in cell signaling: Apoptosis. *Comprehensive Toxicology***.** (2009).

provides the driving force for ATP production. During stress-induced apoptosis, the mitochondrial outer membrane is permeabilized, resulting in release of several proapoptotic factors into the cytosol, including cytochrome c, apoptosis-inducing factor (AIF), endonuclease G, Smac/DIABLO, and Omi/HtrA2 (Du et al., 2000; Li et al., 2001; Susin et al., 1999; Suzuki et al., 2001; Verhagen et al., 2000). Following MOMP, the released cytochrome c binds to Apaf-1, which consists of an N-terminal nucleotide binding domain (NBD), CARD and a series of C-terminal WD-40 repeats. Apaf-1, upon binding to cytochrome c through its WD-40 repeats, binds to ATP/dATP and undergoes oligomerization *via* its NBD into a heptameric complex termed the apoptosome (Acehan et al., 2002; Li et al., 1997). These interactions expose the CARD domain of Apaf-1, thus making it competent to bind the prodomain of caspase-9. Procaspase-9 once recruited to the apoptosome is activated and undergoes self-processing in the linker region between the large and small subunits (Srinivasula et al., 1998). Active caspase-9 activates effector caspases-3 and -7, which in turn cleaves more than 500 intracellular substrates thus leading to apoptosis (Li et al., 1997; Walsh et al., 2008) (Figure 1.3).

The activated caspases are further regulated by a conserved IAP family of proteins like XIAP. XIAP consists of three BIR domains (BIR1-3) and a C-terminal RING domain (Duckett et al., 1996; Uren et al., 1996). The BIR domains have distinct functions in inhibiting different caspases. The BIR3 domain of XIAP

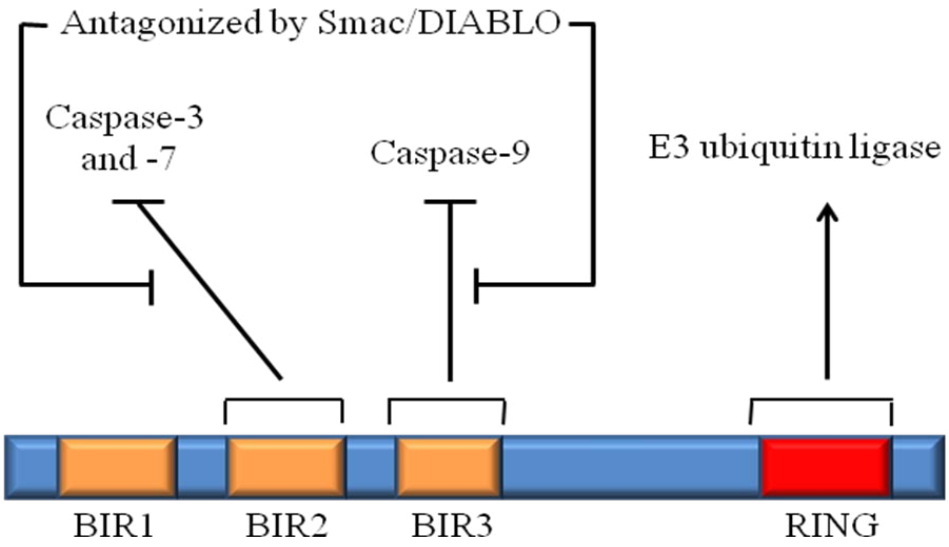


Figure 1.4 Functional domains of XIAP

XIAP has three BIR domains two of which have distinct functions in inhibiting different caspases. XIAP inhibits caspases-3 and -7 *via* its BIR2 domain and an adjacent linker region, while caspase-9 is inhibited by the BIR3 domain. The RING domain on the C-terminus of XIAP has E3 ubiquitin ligase activity. Smac/Diablo antagonizes XIAP by displacing the bound caspases through interaction with the BIR2 and BIR3 domains of XIAP. The figure is modified from Holcik and Korneluk (2001).

binds exclusively to processed caspase-9 through a motif located on the N-terminus of the freshly cleaved small subunit, thereby potently inhibiting its activity (Huang et al., 2001; Srinivasula et al., 2001). With respect to the effector caspases-3 and -7, the linker region located between the BIR1 and BIR2 domains of XIAP plays an important role in binding and inhibiting the caspase activity. This linker region binds into the active site of effector caspases-3 and -7, in the opposite orientation to a normal substrate, thereby preventing cleavage of the linker and access to substrates (Chai et al., 2001; Huang et al., 2001; Riedl et al., 2001) (Figure 1.4). XIAP inhibition of caspases is reversible and can be displaced through binding of IAP antagonists, such as Smac/DIABLO. Smac/DIABLO contains 239 amino acids, but its N-terminal 55 residues encode the mitochondrial-targeting sequence, which is proteolytically removed upon importation into mitochondria. This cleavage results in the exposure of four hydrophobic amino acids, Ala-Val-Pro-Ile, at the N-terminus of mature Smac (Du et al., 2000; Verhagen et al., 2000). The N-terminal Ala of the IAP binding motif (IBM) binds into a hydrophobic pocket in the BIR2 and BIR3 domains of XIAP (Liu et al., 2000; Srinivasula et al., 2000). Smac/DIABLO, through its IBM, antagonizes XIAP by displacing the bound caspases. The N-terminus of the caspase-9 small subunit and the IBM of Smac/DIABLO compete for the hydrophobic pocket in the BIR3 domain, decreasing the ability of XIAP to bind and inhibit caspase-9 (Wu et al., 2000). Similarly, XIAP inhibition of caspase-3 is

also neutralized by the interaction of Smac/DIABLO to the BIR2 domain (Chai et al., 2000) (Figure 1.4).

1.3 BCL-2 family members.

BCL-2 family members help maintain mitochondrial integrity and regulate apoptosis, either by inhibiting or promoting MOMP. There are, at least, twenty mammalian Bcl-2 members, and they are divided into antiapoptotic and proapoptotic members. The antiapoptotic BCL-2 family members include BCL-2, BCL-XL, MCL-1, BCL-W, and A1, which serve as effective barriers to the intrinsic pathway and are characterized by the presence of four α-helical BCL-2 homology (BH) domains (BH1-4), with the exception of MCL-1 (BH1-3) and A1 (BH1-2). The proapoptotic members are subdivided into two classes: the BH3-only members (BID, BIM/BOD, BAD, NOXA, PUMA, BBC3, BMF, HRK/DP5, BIK/BLK/NBK, and MULE) and the multidomain (BH1-3) members (BAX, BAK, and BOK/MTD) (Youle and Strasser, 2008) (Figure 1.5).

Two multidomain proapoptotic members, BAX and BAK, are essential for MOMP, leading to the completion of apoptosis. In the case of BAX, the induction of apoptosis and the associated change in its cellular localization reflects the ability to undergo a conformational change from a soluble to an integral membrane conformation. BAX is composed of 9 alpha helices separated by rather unstructured loops. Two central helices (helix α5, and to a lesser extent helix α6)

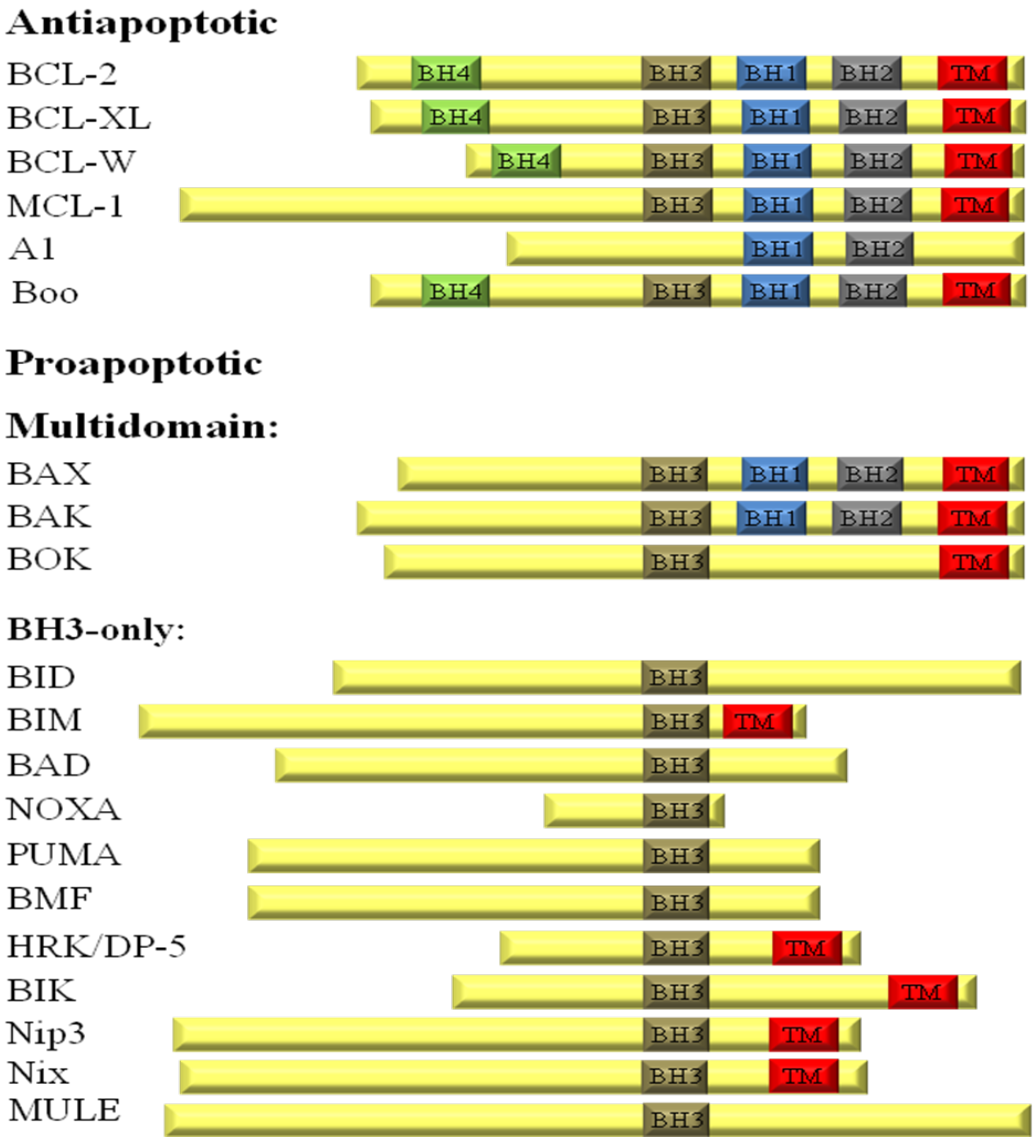


Figure 1.5 Classification of BCL-2 family members

Bcl-2 family members are divided into anti- or proapoptotic family members. Antiapoptotic Bcl-2 family members are characterized by the presence of four BH domains with the exception of MCL-1 and A1. The proapoptotic BCL-2 family members are further subdivided into two subfamilies: the multidomain members, which are characterized by the presence of BH1-3 domains, and the BH3-only members.

are mostly hydrophobic and constitute the core of the protein. The other 7 helices have amphipathic properties and keep their hydrophilic residues exposed to the exterior. This organization enables the protein to remain soluble in its native cytosolic conformation (Suzuki et al., 2000). BAX appears to exist as a monomer in the cytosol rather than being bound to any antiapoptotic BCL-2 family members (Hsu and Youle, 1998). During apoptosis, a major conformational change occurs, wherein several domains, formerly hidden in the native conformation, become exposed. Its C-terminal transmembrane domain (helix α9) is first exposed, resulting in translocation of BAX to mitochondria. BAX then inserts into the OMM and undergoes homooligomerization (Mikhailov et al., 2003; Nechushtan et al., 1999; Suzuki et al., 2000). The N-terminus of BAX (helix α1) is also exposed during its targeting to mitochondria (Cartron et al., 2003). On the other hand, BAK is normally localized to the OMM, but during apoptosis BAK similarly undergoes a conformational change that exposes its N-terminus and facilitates oligomerization (Griffiths et al., 1999).

The antiapoptotic BCL-2 family members are critical for regulating MOMP by inhibiting the multidomain proapoptotic members, BAX and BAK to ensure mitochondrial integrity. BAX is inhibited by BCL-2, BCL-XL, MCL-1, and BCL-W, while BAK is inhibited exclusively by MCL-1 and BCL-XL (Youle and Strasser, 2008). Similarly, the BH3-only proteins also possess different affinities for the antiapoptotic BCL-2 family members. BIM, BID, and PUMA

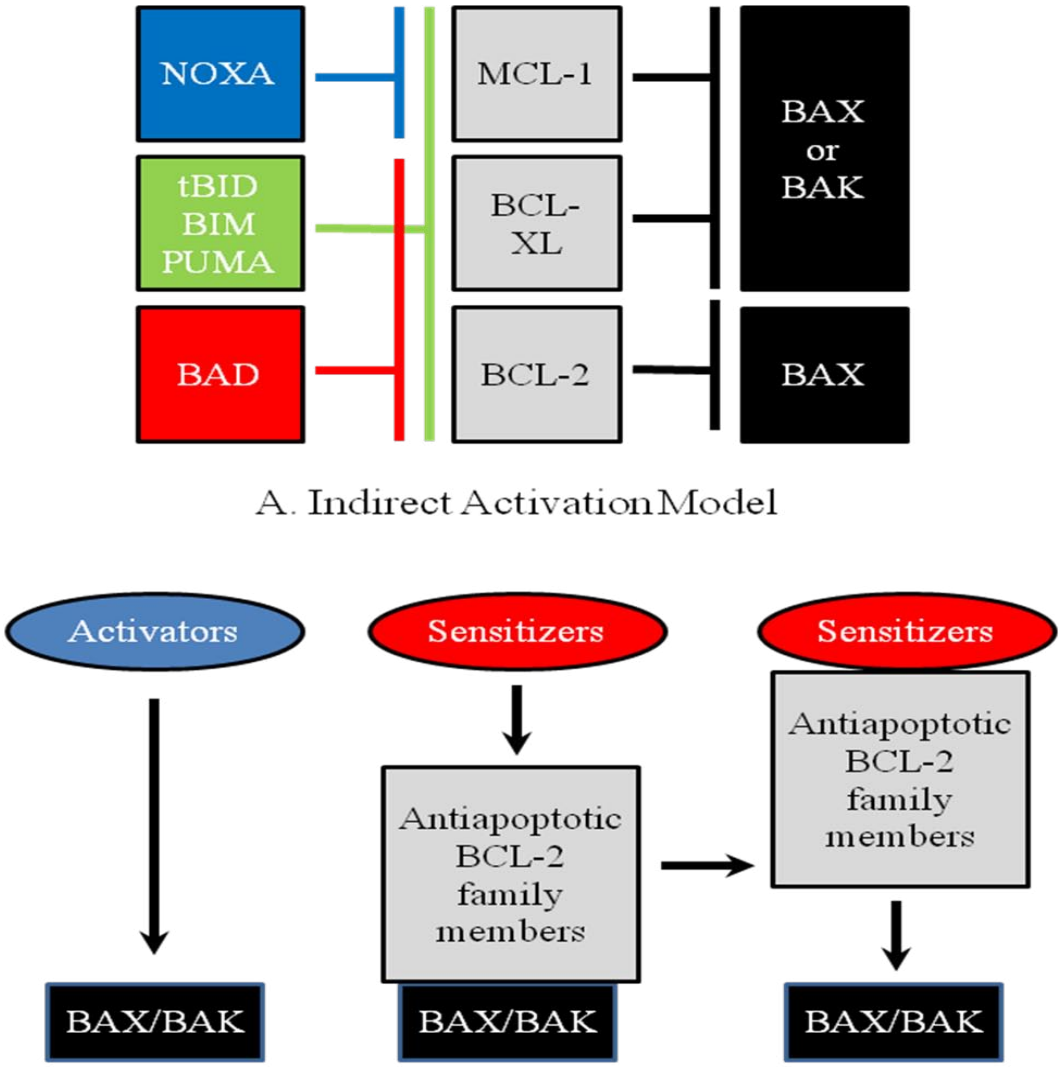


Figure 1.6 Models of BAX/BAK activation by BH3-only proteins

(A) Indirect activation model: BH3-only proteins antagonize BCL-2, BCL-XL, and MCL-1, which restrain BAX and BAK. (B) Direct activation model: BH3-only proteins serve as either direct "activators" of BAX and BAK, or they function as "sensitizers" by displacing the activators from antiapoptotic BCL-2 family members such as BCL-2, BCL-XL, and MCL-1. The figure is modified from Chapter 2. Alterations in cell signaling: Apoptosis. *Comprehensive Toxicology***.** (2009).

bind to all antiapoptotic members. By contrast, BAD and NOXA selectively inhibits BCL-2/ BCL-XL/BCL-W and MCL-1/A1, respectively (Figure 1.6). The BH3-only proteins activate BAX and BAK through two models known as the "indirect activation and direct activation models". In the indirect activation model, BAX and BAK are inhibited by the antiapoptotic BCL-2 family members and the BH3-only proteins induce BAX/BAK activation and apoptosis by neutralizing the antiapoptotic BCL-2 family members. The hydrophobic binding groove of the antiapoptotic BCL-2 family members is occupied by the BH3 domain of the BH3-only proteins (Chipuk and Green, 2008; Youle and Strasser, 2008) (Figure 1.6). By contrast, in the direct activation model, the BH3-only proteins are classified as either "activators" or "sensitizers" (Cheng et al., 2001; Letai et al., 2002). Activators (tBID, BIM, and PUMA) are proposed to directly interact (*via* their BH3 domains) with BAX or BAK, thereby promoting BAX/BAK activation. Indeed, it has been reported that tBID can induce BAX pore formation in large unilamellar vesicles (Kuwana et al., 2002). Sensitizer BH3-only proteins, on the other hand, lack the ability to interact directly with BAX or BAK, but can bind to antiapoptotic BCL-2 family members, thereby displacing the activator BH3-only proteins and allowing these BH3 proteins to activate BAX and BAK (Cheng et al., 2001; Letai et al., 2002) (Figure 1.6).

1.4. MCL-1 (myeloid cell leukemia-1)

The *mcl-1* gene was originally identified as an early gene induced during differentiation of human myeloid leukemia cells (Kozopas et al., 1993). The human *mcl-1* gene is located on chromosome 1q21 and comprises three exons. Alternative splicing gives rise to two distinct MCL-1 mRNAs, MCL-1L and MCL-1S, containing or lacking exon 2, respectively (Bingle et al., 2000). The absence of exon 2 in MCL-1S causes exon 3 sequences to be translated in a different reading frame, so that the C-terminal transmembrane (TM) domain in MCL-1L is not included in MCL-1S (MCL-1S/ΔTM). MCL-1S contains 271 amino acid residues and a BH3 domain only, whereas MCL-1L comprises 350 amino acid residues and has BH (1-3) domains (Kozopas et al., 1993), as well as a C-terminal TM domain that localizes MCL-1L to the OMM (Yang et al., 1995). Interestingly, these distinct MCL-1 isoforms have opposing functions in that MCL-1L is antiapoptotic, whereas MCL-1S possesses proapoptotic function similar to other BH3-only proteins (Bingle et al., 2000). The N-terminal regions of MCL-1 contain two PEST domains, rich in proline (P), glutamate (E), serine (S), and threonine (T) residues, and are thought to induce rapid protein turnover (Rechsteiner and Rogers, 1996; Rogers et al., 1986) and MCL-1 has a short half-life in cells (Craig, 2002; Nijhawan et al., 2003).

1.4.1 MCL-1 functions

Gene-ablation studies have revealed physiological roles for MCL-1 which are markedly divergent from those of other antiapoptotic BCL-2 family members. MCL-1 deficient mice display peri-implantation lethality, which is apparently not due to increased apoptosis, suggesting that MCL-1 plays a distinct role during implantation (Rinkenberger et al., 2000). MCL-1 is also essential for the development and maintenance of B and T lymphocytes, as deletion of MCL-1 during early lymphocyte differentiation increases apoptosis and arrests development, while deletion of MCL-1 in mature B and T lymphocytes results in rapid apoptosis (Opferman et al., 2003). Furthermore, deletion of MCL-1 in early hematopoietic progenitors leads to the severe loss of bone marrow, indicating an indispensable role for MCL-1 in hematopoietic stem cell survival (Opferman et al., 2005). As is the case with other antiapoptotic BCL-2 family members, MCL-1 is primarily localized to the OMM and promotes cell survival by blocking MOMP *via* interaction with and neutralization of the multidomain proapoptotic BCL-2 family members including BAK (Shimazu et al., 2007). Like other antiapoptotic BCL-2 family members, MCL-1 interacts with BIM, tBID, BIK, and PUMA, but also binds to NOXA and BAK (Chen et al., 2005a; Clohessy et al., 2006; Willis et al., 2005). In fact, the BH3-only protein NOXA binds almost exclusively to MCL-1. The precise molecular mechanism by which MCL-1 promotes cell survival is not completely understood. The most plausible mechanism of antiapoptotic action of MCL-1 is that MCL-1 sequesters BAK on the OMM, leading to inhibition of

BAK oligomerization and MOMP (Shimazu et al., 2007; Willis et al., 2005). Upon apoptotic stimuli, Bik, tBID, and NOXA can selectively disrupt MCL-1/BAK interaction to displace BAK from MCL-1, leading to BAK oligomerization and MOMP (Clohessy et al., 2006; Shimazu et al., 2007; Willis et al., 2005).

1.4.2 Regulation of MCL-1

MCL-1 has a short half-life and is highly regulated at the transcriptional, post-transcriptional, translational, and post-translational levels. MCL-1 is regulated at the transcriptional level *via* the transcription factors SRF/ETS, STAT3, CREB, and PU.1 (Epling-Burnette et al., 2001; Nijhawan et al., 2003; Puthier et al., 1999; Wang et al., 1999), and at the post-transcriptional level through alternative splicing leading to accumulation of two distinct MCL-1 mRNA encoding MCL-1L and MCL-1S isoforms with opposing functions (Bingle et al., 2000). MCL-1 is also regulated at the translational level by micro-RNAs through a mir-29b binding in the 3'-UTR of MCL-1 mRNA (Mott et al., 2007). Mir-29b directly inhibits expression of MCL-1 by binding to its target sequence. The RNA binding protein, CUGBP2, can also bind to the 3'-UTR of MCL-1 and inhibit its translation (Subramaniam et al., 2008). Post-translational regulation of MCL-1 is mediated by a complex interplay involving three kinases (ERK, JNK, and GSK-3β) and at least two E3 ubiquitin ligases (MCL-1 ubiquitin

ligase E3 (MULE) and ß-TrCP) (Ding et al., 2007; Domina et al., 2004; Maurer et al., 2006; Morel et al., 2009; Mott et al., 2007; Nijhawan et al., 2003). ERK-mediated phosphorylation of human MCL-1 at Thr-163 prolongs its half-life (Domina et al., 2004), although recent studies by Davis and colleagues indicate that JNK phosphorylates mouse MCL-1 at Thr-144 (analogous to Thr-163 in human MCL-1), which in turn enhances its phosphorylation by GSK-3β at Ser-140 (Morel et al., 2009). GSK-3ß-mediated phosphorylation of mouse/human MCL-1 at Ser-140/Ser-159 then promotes its ubiquitination by E3 ligases and subsequent degradation by the 26S proteasome (Maurer et al., 2006; Morel et al., 2009).

1.5 p38 MAPKs

MAPKs are a family of serine/threonine protein kinases, which are activated in response to extracellular stimuli including mitogens, growth factors, and environmental stress, and regulate various cellular activities such as gene expression, mitosis, differentiation, proliferation, and cell survival/apoptosis. Conventional MAPKs consist of three family members: ERKs, JNKs, and p38 MAPKs and each family member has its own subfamilies: ERKs (ERK1 and ERK2), JNKs (JNK1, JNK2, and JNK3), and p38 MAPKs (p38α MAPK, p38ß MAPK, p38γ MAPK, and p38δ MAPK) (Chang and Karin, 2001; Davis, 2000; Johnson and Lapadat, 2002). An additional MAPK, termed ERK5, has been

cloned (Zhou et al., 1995), and ERK5 is a member of a larger MAPK family that also includes ERK7 and ERK8 (Abe et al., 1999). However, the functions and activation pathways for ERK7 and ERK8 are not fully characterized. The MAPKs share a common Tyr-Xaa-Thr dual phosphorylation site in their regulatory loops (Hanks and Hunter, 1995). MAPKs are activated through activation pathway, comprising a MKKK, MAPK kinase (MKK), and MAPK (Wagner and Nebreda, 2009).

1.5.1 Identification of p38 MAPKs

Among all p38 MAPKs isoforms, p38α is the best characterized and is expressed in most cell types. p38α was first isolated as 38 kDa protein that underwent tyrosine phosphorylation in response to endotoxin treatment and hyperosmolarity shock (Han et al., 1994), and three additional isoforms were then identified: p38ß (Jiang et al., 1996), p38γ (SAPK3/ERK6) (Lechner et al., 1996; Mertens et al., 1996), and p38δ (SAPK4) (Goedert et al., 1997; Jiang et al., 1997). Of these, p38α and p38ß are ubiquitously expressed (Jiang et al., 1996), while the others are differently expressed in different tissues. p38γ is predominantly expressed in skeletal muscle (Lechner et al., 1996; Li et al., 1996), and p38δ is mainly found in testis, pancreas, and small intestine (Goedert et al., 1997). All isoforms have a Thr-Gly-Tyr (TGY) dual phosphorylation motif in the regulatory loop between the kinase subdomains (Hanks and Hunter, 1995). p38α and p38ß

have high sequence homology (75%) and share sensitivity to pharmacological inhibitors such as SB203580, whereas p38γ and p38δ have 60% homology and are resistant to SB203580 (Eyers et al., 1999).

1.5.2 Activation and regulation of p38 MAPKs

p38 MAPKs are activated by a variety of stimuli such as UV irradiation, heat, inflammatory cytokines, and growth factors. Upon stimulation, p38 MAPKs are activated through dual phosphorylation of their TGY motif by the MKKs, MKK3 and MKK6 (Enslen et al., 1998). Phosphorylated p38 MAPK then goes through a characteristic conformational change that alters the alignment of the two kinase halves (N- and C-terminal domains), enhancing access to substrates, and increasing its enzymatic activity (Bellon et al., 1999). MKK3 and MKK6 are activated *via* phosphorylation by MKKKs. Several MKKKs have been implicated in the regulation of p38 MAPK pathways, including mixed lineage protein kinases (MLKs), ASK1, TAK1, and some members of MEK kinases (MKKs) (Cheung et al., 2003; Ichijo et al., 1997b; Kyriakis and Avruch, 2001; Yamaguchi et al., 1995). MKKKs responsible for activating p38 MAPKs appear to be cell-type and stimulus-specific. p38 MAPKs can also be activated by two alternative MKK-independent mechanisms. In the first, phosphorylation of p38α is achieved through autophosphorylation and activation following interaction with TAK1-binding protein 1 (TAB1) (Ge et al., 2002a). Another MKK-independent pathway

has been reported in T cells stimulated through the T cell antigen receptor (TCR). In this mechanism, p38α is activated by the TCR-stimulated tyrosine kinase ZAP-70, which phosphorylates p38α at a noncanonical Tyr323 site (Salvador et al., 2005). Once activated, p38 MAPKs undergo a sequence of phosphorylation to act on several downstream substrates, including protein kinases or transcription factors, resulting in a wide variety of responses. MAP kinase-activated protein kinase 2 (MAPKAPK2) was identified as a p38α substrate (Rouse et al., 1994). Moveover, activated MAPKAPK2 phosphorylates various substrates including heat shock protein 27 (HSP27) (Stokoe et al., 1992), lymphocyte-specific protein 1 (LSP1) (Huang et al., 1997), cAMP response element-binding protein (CREB) (Tan et al., 1996), activating transcription factor 1 (ATF1) (Tan et al., 1996), serum response factor (SRF) (Heidenreich et al., 1999), and tyrosine hydroxylase (Thomas et al., 1997).

Like all MAPKs, p38 MAPKs are downregulated through dephosphorylation by phosphatases. Several phosphatases interact with and inactivate p38 MAPKs, including serine/threonine protein phosphatase type 2Calpha (PP2Calpha) and protein tyrosine phosphatase (PTPase) (Takekawa et al., 2000; Takekawa et al., 1998). Other studies have suggested that MAPK phosphatase (MKP)-1, -4, and -5 can dephosphorylate p38α and p38ß (Camps et al., 1998; Muda et al., 1996). Structural studies indicate that a p38 MAPK-specific pyridinyl imidazole inhibitor, SB203580, binds to the active site of both

phosphorylated (active) and unphosphorylated (inactive) p38α and p38ß isoforms in an ATP-competitive manner (Tong et al., 1997). SB203580 binds to an aryl-specificity pocket normally occupied by the adenine ring of ATP.

1.5.3 Biological consequences of p38 MAPK activation

p38 MAPK signaling has been implicated in numerous cellular responses from inflammation, cell cycle, and cell death to development, cell differentiation, senescence, and tumorigenesis. However, the precise roles of p38 MAPKs in many of these processes remain unclear. p38α was first recognized for its role in inflammation in regulating the biosynthesis of proinflammatory cytokines such as interleukin (IL)-1 and TNFα in endotoxin-stimulated monocytes (Lee et al., 1994). Subsequently, it was found to be involved in regulating the production of IL-8 in response to IL-1 or osmotic shock and the production of IL-6 in response to TNFα (Beyaert et al., 1996; Shapiro and Dinarello, 1995). p38α is also essential for embryogenesis, as genetic knockout of p38α results in embryonic lethality due to its importance in erythropoiesis and placental angiogenesis (Adams et al., 2000; Beyaert et al., 1996; Tamura et al., 2000). Gene-ablation studies indicate that p38α is also involved in the proliferation of lung stem and progenitor cells (Ventura et al., 2007) and in the differentiation of myoblasts into multinucleated myotubes (Perdiguero et al., 2007). p38α can also regulate cell cycle progression, both at the G1/S and G2/M transitions, by downregulating cyclins and

upregulating cyclin-dependent kinase (CDK) inhibitors (Ambrosino and Nebreda, 2001).

p38α is also involved in tumorigenesis. Genetic modification indicates that p38α acts as a tumor suppressor. For instance, constitutive activation of p38 MAPK, achieved through overexpression of it upstream activators, MKK3 and MKK6, results in senescence and suppression of tumorigenesis in several cancer cell models (Haq et al., 2002; Wang et al., 2002), and MKK3 and MKK6 double knockout fibroblasts are more susceptible to increase tumorigenesis (Brancho et al., 2003). Many studies have reported that p38 MAPK signaling is involved in apoptosis. However, while p38 MAPK signaling has been shown to promote cell death in some cell lines (Porras et al., 2004; Sarkar et al., 2002), in others it enhances survival (Liu et al., 2001a; Park et al., 2002). Thus, the role of p38 MAPK signaling in apoptosis is cell-type specific and stimulus-dependent.

1.6 Dissertation objectives

TRAIL has emerged as a promising anticancer therapy, due to its remarkable capacity to induce apoptosis in cancer cells with little to no toxicity to normal cells (Falschlehner et al., 2009). Therefore, recombinant human TRAIL (rhTRAIL) and agonistic monoclonal antibodies to DR4 and DR5 are being considered for therapeutic use. Phase I trials have established the safety and tolerability of these TRAIL agonists in patients, and Phase II trials are currently

evaluating the therapeutic efficacy of TRAIL agonists as single agents or in combination with established cancer therapeutics. Many cancers, however, exhibit or acquire resistance to TRAIL. Thus, understanding the underlying mechanisms that mediate TRAIL resistance is necessary to provide more effective cancer treatment.

The objective of my dissertation was to understand the primary mechanisms responsible for TRAIL resistance in human prostate cancer cells, with a particular focus on the role of p38 MAPKs in this process. I hypothesized that TRAIL activates a TAK1→MKK3/MKK6→p38 pathway that transcriptionally upregulates the expression of MCL-1 and suppresses BAK activation, MOMP, and cell death, despite caspase-8 activation and robust BID cleavage. Disruption of the p38 MAPK signaling pathway downregulated MCL-1 and sensitized cells to TRAIL-induced MOMP and apoptosis. However, reactive oxygen species (ROS), generated by injured mitochondria, activated a secondary JNK pathway that upregulated MCL-1 expression and afforded partial protection from death. Thus, this work demonstrates for the first time that stress kinases activated at distinct steps in the extrinsic pathway mediate TRAIL resistance through maintenance of MCL-1 expression levels.

Chapter 2. Materials and Methods

2.1 Reagents and antibodies

SB203580 (cat. #1020) was obtained from Tocris Bioscience (Ellisville, MO), and 5Z-7-oxozeaenol (cat. #NP-009245) was purchased from AnalytiCon Discovery GmbH (Potsdam, Germany). TAT-TI-$JIP_{153–163}$ (cat. #420134) and U0126 (cat. #662005) were obtained from Calbiochem (Gibbstown, NJ). Antibodies to p-p38 MAPK (Thr-180/Tyr-182; cat. #9211), p-ERK (Thr-202/Tyr-204; cat. #9101), p-JNK (Thr-183/Tyr-185; cat. #9251), p-MAPKAPK2 (pThr-222; cat. #3316), p-AKT (Ser-473; cat. #9271), p-MKK3/6 (Ser-189/207; cat. #9231), p-TAK1 (Thr-184/Tyr-187; cat. #4531), TAK1 (cat. #4505), BCL-2 (cat. #2876), BCL-x_L (cat. #2762), BID (cat. #2002), myc tag (cat. #2276), EGFP (cat. #2555), and active caspase-3 (cat. #9662) were purchased from Cell Signaling Technology (Danvers, MA). FLIP antibody (cat. #ALX-804-127-C100) was obtained from Alexis Biochemicals (San Diego, CA), and caspase-8 and Smac/DIABLO antibodies were generously provided by Dr. X.-M. Sun and Prof. G. M. Cohen (MRC Toxicology Unit, Leicester, UK). Antibodies to FADD (cat. #610399) and cytochrome c (cat. #556433) were purchased from BD Biosciences (San Jose, CA), and antibodies to MCL-1 (cat. #sc-12756) and actin (cat. #CP01-100UL) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and Oncogene Research Products (Cambridge, MA), respectively. DR5 antibody (cat.

#2019) was obtained from ProSci Incorporated (Poway, CA), and antibodies to FLAG (cat. #F3165) and Hemagglutinin (cat. #MMS-101P) tags were purchased from Sigma-Aldrich (Saint Louis, MO) and Covance (Princeton, NY), respectively. RFP/Cherry antibody (cat. #PM005) was obtained from MBL International (Woburn, MA).

2.2 Cloning

The human *MCL-1* cDNA encompassing the entire coding region (1.1 kb) was amplified by reverse transcription-PCR (RT-PCR) with the following two primers (MCL1F: 5'-CCCGAATTCATGTTTGGCCTCAAAAGAAACGC-3'; MCL1R: 5'-CCGAGATCTCTATCTTATTAGATATGCCAAACC-3'), digested, and cloned into the *Eco*RI-*Bgl*II sites (underlined) of pEGFP-C1 (Clontech, Mountain View, CA). The cDNAs of human *BAD* and *NOXA* were obtained by RT-PCR with the following primers (BADF: 5'-CCGCTCGAGCTATGTTCCA GATCCCAGAGTTTG-3'; BADR: 5'-CGCGGATCCTCACTGGGAGGGGGC GGAG-3'; NOXAF: 5'-CCGCTCGAGCTATGCCTG GGAAGAAGGCGCG-3'; NOXAR: 5'-CGCGGATCCTCAGGTTCCTGAGCAGAAG AG-3'), digested, and cloned into the *Xho*I-*Bam*HI sites (underlined) of pCherry-C1 (Clontech, Mountain View, CA). For the generation of shRNAs, oligonucleotides to *MCL-1* (sense: 5'-GATCCCCAGTCCGATTACCGCGTTTCTTCAAGAGAGAAACG CGGTAATCGGACTTTTTTTC-3'; antisense: 5'-TCGAGAAAAAAAGTCCG

ATTACCGCGTTTCTCTCTTGAAGAAACGCGGTAATCGGACTGGG-3') or a scrambled control (sense: 5'-GATCCCCACCGTCGATTTCACCCGGGTTCA AGAGACCCGGGTGAAATCGACGGTTTTTTTC-3'; antisense: 5'-TCGAGA AAAAAACCGTCGATTTCACCCGGGTCTCTTGAACCCGGGTGAAATCGA CGGTGGG-3') were annealed to generate sticky ends (underlined) and immediately cloned into the *Bam*HI-*Xho*I sites of pSuper.retro.puro (OligoEngine, Seattle, WA). The boxed sequence corresponds to the 9-nt hairpin that is generated following transcription.

A cDNA encoding p38α (kindly provided by Dr. Kevin N. Dalby, The University of Texas at Austin, Austin, TX) was subcloned into pcDNA6 with an N-terminal myc tag (Invitrogen, Carlsbad, CA), and a kinase-dead (D168A) mutant was subsequently incorporated by site-directed mutagenesis. A constitutively-active mutant of p38α (D176A/ F327S; obtained from Prof. David Engelberg, Hebrew University, Israel)(Askari et al., 2007) was subcloned into the *Xho*I-*Bam*HI sites of the pEGFP-C1 and pCherry-C1 vectors (Clontech, Mountain View, CA), and an SB203580-resistant mutation (T106M) was incorporated by site-directed mutagenesis (Eyers et al., 1999). Dominant-negative mutants of TAK1 (K63A), MKK3 (S189A/T193A), and MKK6 (S207A/T211A) were generously provided by Dr. Xin Lin (University of Texas, M. D. Anderson Cancer Center, Houston, TX) and Dr. Jiahuai Han (Scripps Research Institute, La Jolla,

CA) (Blonska et al., 2005; Ge et al., 2002b). pEBB-ubiquitin-Smac/DIABLO, which is designed to express a mature form of Smac in the cytosol, was provided by Dr. Colin S. Duckett (University of Michigan Medical School, Ann Arbor, MI) (Hunter et al., 2003). Wild-type caspase-9 was cloned into the FG9 lentiviral vector (kindly provided by Dr. Casey W. Wright, UT-Austin, Austin, TX), and a dominant-negative mutation (C287A) was incorporated by site-direct mutagenesis.

2.3 Cell culture and Transfections

DU145 prostate cancer cells were grown in RPMI-1640, supplemented with 5% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 5% Fetalplex (Gemini Bio-products, West Sacramento, CA), 1% penicillin-streptomycin (100 units/mL), and 2 mM glutamine. Cells were maintained at 37°C in humidified air containing 5% CO_2 and were routinely passaged every 3 days. For transient transfections, cells were transfected with 1 μg/mL plasmid DNA using *Trans*IT®-LT1 transfection Reagent (Mirus Bio, Madison, WI). For stable transfections, cells were selected for 10 days in 1 μg/mL of puromycin and then isolated as individual clones.

2.4 Quantitation of apoptosis

Cells were harvested by trypsinization, washed with PBS, and resuspended in annexin-V binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM

$CaCl_2$) containing annexin-V-fluorescein isothiocyanate (FITC) and propidium iodide (PI; Roche Applied Sciences, Indianapolis, IN). Annexin V was expressed, labeled with FITC, and purified in-house. FITC- and/or propidium iodide-labeled cell populations were analyzed by flow cytometer (Beckman-Coulter, Fullerton, CA).

2.5 DEVDase assay

Cells were pretreated with SB203580 (50 μM) for 2 h, cotreated with TRAIL (500 ng/ml) for an additional 8 h, and collected for analysis. The cells were then washed twice with PBS, resuspended in lysis buffer (50 mM Tris, pH7.5, 1 mM EDTA, 10 mM EGTA, 10 μM digitonin), and incubated for 10 min at 37°C. Cytosolic fractions were obtained by centrifugation at 15,000 x g for 10 min and were subsequently incubated for 30 min at 37°C with an equal volume of assay buffer (20 mM HEPES, pH 7.4, 100 mM NaCl, 0.05% NP-40, 5 mM $MgCl_2$) containing the fluorescent substrate, Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (DEVD-AMC). DEVDase activities were measured ($\lambda_{ex}/\lambda_{em}$ = 360/450 nm) in a 96-well plate format using a Wallac Victor^3 1420 Multilabel counter (Waltham, MA). To confirm the effectiveness of our dominant-negative (DN) caspase-9 (C287A) construct, lysates were prepared from cells stably expressing caspase-9-DN and activated by adding cytochrome c (10 μM), dATP (2 mM), and $MgCl_2$ (2 mM). Following a 30 min incubation at

37°C, DEVD-AMC was added and all samples were assayed for DEVDase activity (Bratton et al., 2000).

2.6 Digitonin-based subcellular fractionation

Cells, treated with TRAIL ± SB203580 (as described above), were assayed for MOMP, essentially as previously described (Milleron and Bratton, 2006). Briefly, cells were washed with PBS and lysed for 10 min on ice in 100 µL of digitonin lysis buffer (75 mM KCl, 1 mM NaH_2PO_4, 8 mM Na_2HPO_4, 250 mM sucrose, 60 µg/mL of digitonin) containing protease inhibitors (1 mM PMSF, 2 µg/mL of aprotinin, 2 µg/mL of leupeptin, 2 µg/mL of pepstatin). The cells were then pelleted by centrifugation at 15,000 x g for 10 min, and the supernatant (cytosol) was obtained. The remaining pellet (mitochondria) was then resuspended in lysis buffer (20 mM Tris-HCl, 135 mM NaCl, 10% glycerol, 50 mM NaF, 5 mM Na_3VO_4, 0.2% NP-40) with protease inhibitors for 20 min on ice. Finally, the supernatant and pellet fractions were immunoblotted with an antibody to cytochrome c.

2.7 Isolation of TRAIL receptor signaling complexes

Cells were pretreated with SB203580 (50 µM) for 2 h, followed by a 30 min treatment with biotinylated TRAIL (bTRAIL; 500 ng/ml). The cells were then washed three times with ice-cold PBS to remove any unbound ligand and

lysed for 30 min on ice in 3 mL of lysis buffer [30 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 10 mM glycerophosphate, 1 mM sodium orthovanadate, 5 mM NaF] with protease inhibitors (1mM PMSF, 2 μg/mL of aprotinin, 2 μg/mL of leupeptin, 2 μg/mL of pepstatin). Lysates were cleared by centrifugation at 13,000 x g for 30 min and bTRAIL complexes precipitated overnight at 4ºC using streptavidin-conjugated to Sepharose™ beads (Amersham Biosciences, Pittsburgh, PA). After overnight incubation, the beads were washed four times with lysis buffer, and 60 μL of 2X Laemmli sample buffer were added to the beads.

2.8 Quantitative RT-PCR

Cells were plated at a density of 0.6 x 10^6 cells. After 24h, the cells were treated with different combination treatments at the indicated time points. Total RNA was extracted using Qiagen RNeasy Mini kit (Valencia, CA), and reverse transcription was performed from 2 μg of total RNA using oligo-dT and AMV reverse transcriptase (Promega, Madison, WI), according to the manufacturer's instructions. The primer sequences were designed as follows: qMCL1F: 5'-AAGCCAATGGGCAGGTC T-3'; qMCL1R: 5'-TGTCCAGTTTCCGAAGCA T-3'; qGAPDHF: 5'-TGCACCACCAACTGCTTAGC-3'; qGAPDHR: 5'-GG CATGGACTGTGGTCATGAG-3'(Juo et al., 1998). Quantitative RT-PCR was performed with SYBR Green dye using an ABI 7900HT (Perkin-Elmer Applied

Biosystems, Foster City, CA), according to the manufacturer's instructions. PCR reactions were performed in triplicate, and the relative amount of *MCL-1* cDNA was calculated by the comparative CT method (Livak and Schmittgen, 2001). CT values obtained for the different samples were normalized to corresponding CT values of GAPDH.

2.9 Western blot analysis

After SDS-PAGE, proteins were transferred to Hybond-N Nitrocellulose (Amersham Biosciences, Pittsburgh, PA). Membranes were blocked in Tris-buffered saline (TBS) containing 5% non-fat dry milk and 0.1% Tween 20 (TBS-T), prior to incubation with the primary antibody for 1 h. The membranes were then washed with TBS-T followed by exposure to the appropriate horseradish peroxidase-conjugated secondary antibody for 1h. Immunostained proteins were visualized on Kodak x-ray film using the enhanced chemiluminescence (ECL) detection system (Amersham Biosciences, Pittsburgh, PA).

2.10 ROS Quantification

To measure ROS, cells were pretreated with SB203580 (50 μM) for 2 h ± zVAD-fmk (50 μM) for 1 h. TRAIL (500 ng/ml) was then added for 8 h and incubated with 2',7'-dichlorodihydrofluorescein diacetate (DCFDA, 10 μM; Molecular Probes, Carlsbad, CA) for 30 min. Excess DCFDA was removed by

washing the cells twice with PBS at room temperature, and labeled cells were then trypsinized, rinsed, and resuspended in PBS. Oxidation of DCFDA to the highly fluorescent 2',7'-dichloro-fluorescein (DCF) is proportionate to ROS generation and was analyzed by flow cytometry. To measure mitochondrial-generated ROS, pHyPer-Mito, a mitochondrial-localized oxidant-activated fluorescent protein, was transfected into cells and cells were treated TRAIL ± SB203580 (as described above). Fluorescence intensity, corresponding to H_2O_2 generated in the mitochondria, was analyzed by flow cytometry, and images were captured by fluorescence microscopy.

2.11 BAK activation assay

To detect activated BAK, control and treated cells were fixed for 5 min in 0.25% paraformaldehyde, washed twice with PBS, and incubated for 30 min at room temperature in a PBS buffer containing 100 μg/mL digitonin and a conformation-specific mouse anti-BAK antibody (1:30; AM03, Calbiochem, Gibbstown, NJ). Cells were then washed, incubated with 0.25 μg of Alexa Fluor 488 goat anti-mouse antibody (Invitrogen, Carlsbad, CA) for 30 min in the dark, washed again, and analyzed by flow cytometry.

Chapter 3. TRAIL-activated p38 MAPK pathway suppresses apoptosis through transcriptional upregulation of *MCL-1* in human prostate cancer cells

3.1 Introduction

Prostate cancer is the most common type of cancer found in American men. As the second leading cause of cancer-related deaths, it has been estimated that there will be about 192,280 new cases of prostate cancer and approximately 27,360 men will die of this disease in 2009 (American Cancer Society, 2009).

A multicellular organism maintains cellular homeostasis in normal tissue compartments and eliminates disordered cells by a controlled cellular mechanism known as apoptosis (Kerr et al., 1972). Apoptosis can be induced by various stimuli, and radiation or chemicals in particular have been used in cancer therapy. There are two signaling pathways that lead to the activation of apoptosis: the extrinsic and intrinsic pathways. The intrinsic pathway, controlled by BCL-2 family members, has a substantial role in chemotherapy and radiation-induced cell death. By contrast, the extrinsic pathway is initiated through apoptotic signal transduction cascades mediated by members of TNF family (Cotter, 2009). Thus, death ligands have been considered as potentially useful new therapeutics. In particular, TRAIL has emerged as a promising anticancer therapy, due to its remarkable capacity to induce apoptosis in cancer cells with little to no toxicity to

normal cells (Falschlehner et al., 2009). Several different agents related to TRAIL (recombinant human TRAIL and agonistic monoclonal antibodies to TRAIL receptors) have been developed and are being tested in human clinical trials. Although these agents are promising, their utility is limited to patients with TRAIL-sensitive tumors. Unfortunately, many tumors, including human prostate tumors, possess or acquire resistance to TRAIL. Over the last decade many studies have proposed the intracellular mechanisms responsible for TRAIL resistance. The precise mechanisms, however, remain unclear, although it is now clear that the resistance can be caused by numerous factors acting at several different steps in the TRAIL signaling pathway. Expression of TRAIL "decoy" receptors, TRAIL-R3 and TRAIL-R4, has been proposed to mediate TRAIL resistance by competing with TRAIL-R1 and TRAIL-R2 for binding to TRAIL (LeBlanc and Ashkenazi, 2003), and at high expression levels, cellular FLICE-like inhibitory protein (c-FLIP) inhibits apoptosis by competing with caspase-8 for binding to FADD (Clarke and Tyler, 2007; Geserick et al., 2008; Irmler et al., 1997). Finally, overexpression of antiapoptotic BCL-2 family members, including BCL-2, BCL-XL, and MCL-1, is often associated with TRAIL resistance (Fulda et al., 2002; Hinz et al., 2000; Ricci et al., 2007), particularly in Type II cells, due to their abilities to prevent MOMP and the release of cytochrome c or Smac/DIABLO (Bratton and Cohen, 2003).

In Type I cells, the apoptotic signal from active caspase-8 is sufficient to activate the downstream effector procaspase-3 and induce apoptosis. However, in Type II cells, there is insufficient activation of procaspase-3—or caspase-3 is inhibited by IAP , such as XIAP—so that the apoptotic signal must be further amplified by engaging the intrinsic pathway (Bratton and Cohen, 2003; Scaffidi et al., 1999). In this instance, caspase-8 cleaves and activates the BH3-only protein BID, which in turn induces MOMP, causing the release of additional apoptogenic proteins, including the IAP antagonist, second mitochondrial activator of caspases Smac/DIABLO and cytochrome c, the latter of which activates yet another caspase-activating complex, known as the Apaf-1•caspase-9 apoptosome (Bratton and Cohen, 2003). Therefore, inhibition of MOMP is able to cause resistance to TRAIL.

In the present study, I have investigated the role of p38 MAPK in the primary mechanisms responsible for TRAIL resistance in human prostate cancer cells.

3.2 Results

3.2.1 TRAIL activates an antiapoptotic TAK1→MKK3/MKK6→p38 MAPK signaling pathway in resistant prostate cancer cells

In an effort to determine the mechanism(s) of TRAIL resistance in human prostate cancer cells, DU145 cells were exposed to recombinant TRAIL and examined for activation of various stress and growth-related kinases (Fig. 3.1A). Notably, p38α and its downstream target, MAPKAPK2, were activated within 2 h of treatment (prior to cell death), whereas other stress kinases such as JNKs were only weakly activated at later time points (Fig. 3.1A). ERKs were constitutively-active in naive cells, but TRAIL did not enhance their activation, nor did it activate AKT/PKB (Fig. 3.1A; data not shown). The importance of p38α activation was evident when cells were cotreated with TRAIL and the selective p38α and p38ß inhibitor, SB203580, as the combination led to a significant increase in apoptosis (Fig. 3.1B). To examine whether the apoptosis was caspase-dependent, the pan-caspase inhibitor zVAD-fmk was used. Cell death induced by the combination of TRAIL and SB203580 was completely prevented by zVAD-fmk, and the cell death was similar to that of untreated control cells (Fig. 3.1B). Consistent with this result (Fig. 3.1B), TRAIL activated the initiator caspase-8 and the effector caspase-3 (as determined by caspase processing and DEVDase activity), particularly in the presence of SB203580 (Fig. 3.1C). Cell death was

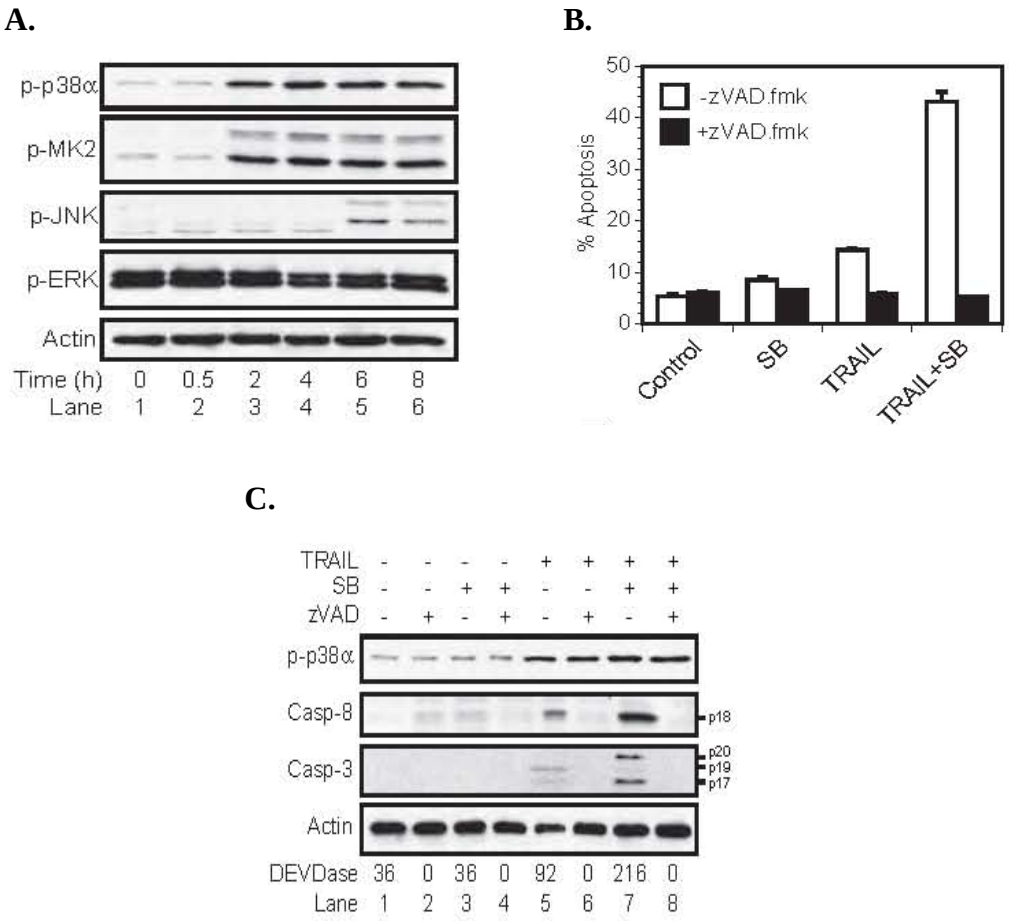


Figure 3.1 Inhibition of TRAIL-activated p38 MAPKs sensitizes cells to TRAIL-induced apoptosis in a caspase-dependent manner.

(*A*) DU145 prostate cancer cells were treated with recombinant TRAIL (500 ng/mL) for 0.5-8 h, and cells were immunoblotted for various active phosphorylated kinases, including p-p38α, p-MK2, p-JNK, and p-ERK. (*B and C*) Cells were treated with TRAIL ± SB203580 (50 μM) ± zVAD-fmk (50 μM) and assayed for cell death by Annexin V/PI staining and flow cytometry. Each bar represents the mean of three separate experiments ± SEM. Cells were also immunoblotted for phosphorylated p38, as well as active caspase-8 (p18 large subunit) and caspase-3 (p20, p19, and p17 large subunits). Caspase-3/7 DEVDase activity was also measured as described in the methods.

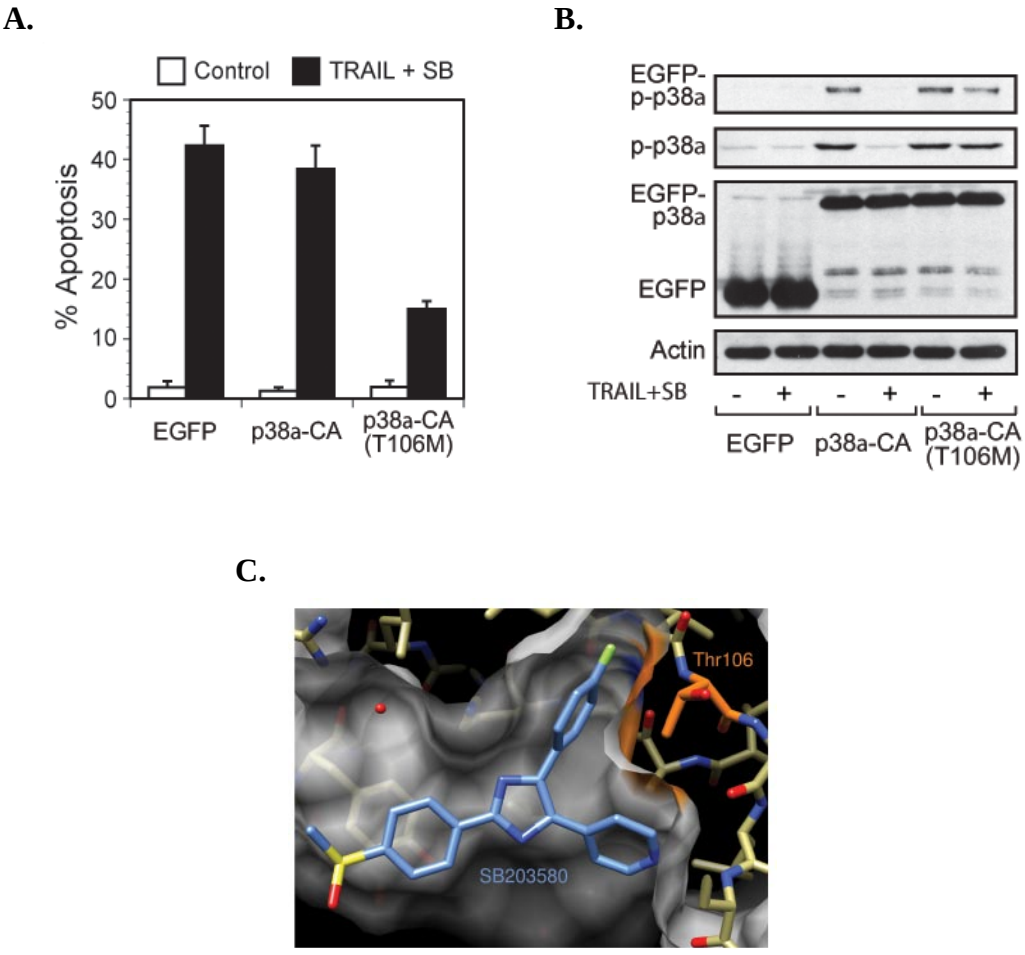


Figure 3.2 TRAIL sensitization is due to specific inhibition of p38 MAPKs.

(*A and B*) DU145 cells were transiently transfected with expression plasmids encoding EGFP, constitutively-active p38α (EGFP-p38α-CA), or p38α-CA containing a T106M mutation [EGFP-p38α-CA (T106M)]. Cells were then exposed to TRAIL for 8 h, stained with Hoechst 33258, and assayed for apoptosis by measuring the percentage of GFP+ cells with condensed nuclei using fluorescence microscopy. Each bar represents the mean of three separate experiments ± SEM. *(C)* Crystal structure of p38α with SB203580 occupying its ATP binding pocket.

fully inhibited by zVAD-fmk; however, this polycaspase inhibitor had no effect on the activation of p38α, further establishing p38α activation as an early upstream event (Fig. 3.1B; Fig. 3.1C, top panel, lanes 5-8). This was an important observation, because although few studies have addressed how TRAIL activates p38α, the adapter protein RIP1 and caspase-8 activity were thought to be essential (Varfolomeev et al., 2005).

To examine whether the cell death caused by cotreatment with TRAIL plus SB203580 was p38α-dependent, cells were transiently transfected with expression plasmids encoding EGFP, constitutively-active (D176A/F327S) p38α (EGFP-p38α-CA), or p38α-CA containing a T106M "gate-keeper" mutation [EGFP-p38α-CA (T106M)]. The sensitization of DU145 cells to TRAIL was due to specific inhibition of p38 MAPKs, as transfection of cells with a constitutively-active form of p38α (CA) largely rescued cells from TRAIL/SB203580-induced cell death, but only when accompanied by a mutation in the "gate-keeper" residue (T106M), which lowers the affinity of SB203580 for the ATP-binding pocket (Fig. 3.2A, C) (Eyers et al., 1999). EGFP-p38α-CA undergoes autophosphorylation and phosphorylates endogenous p38α, but is sensitive to SB203580, whereas EGFP-p38α-CA (T106M) is only weakly inhibited by SB203580 (Fig. 3.2B).

Since TNF reportedly activates p38α through the activation of TAK1 or ASK1 (Cheung et al., 2003; Ichijo et al., 1997a; Tobiume et al., 2001; Wang et al., 2001), we next sought to determine if either of these MKKKs was essential for

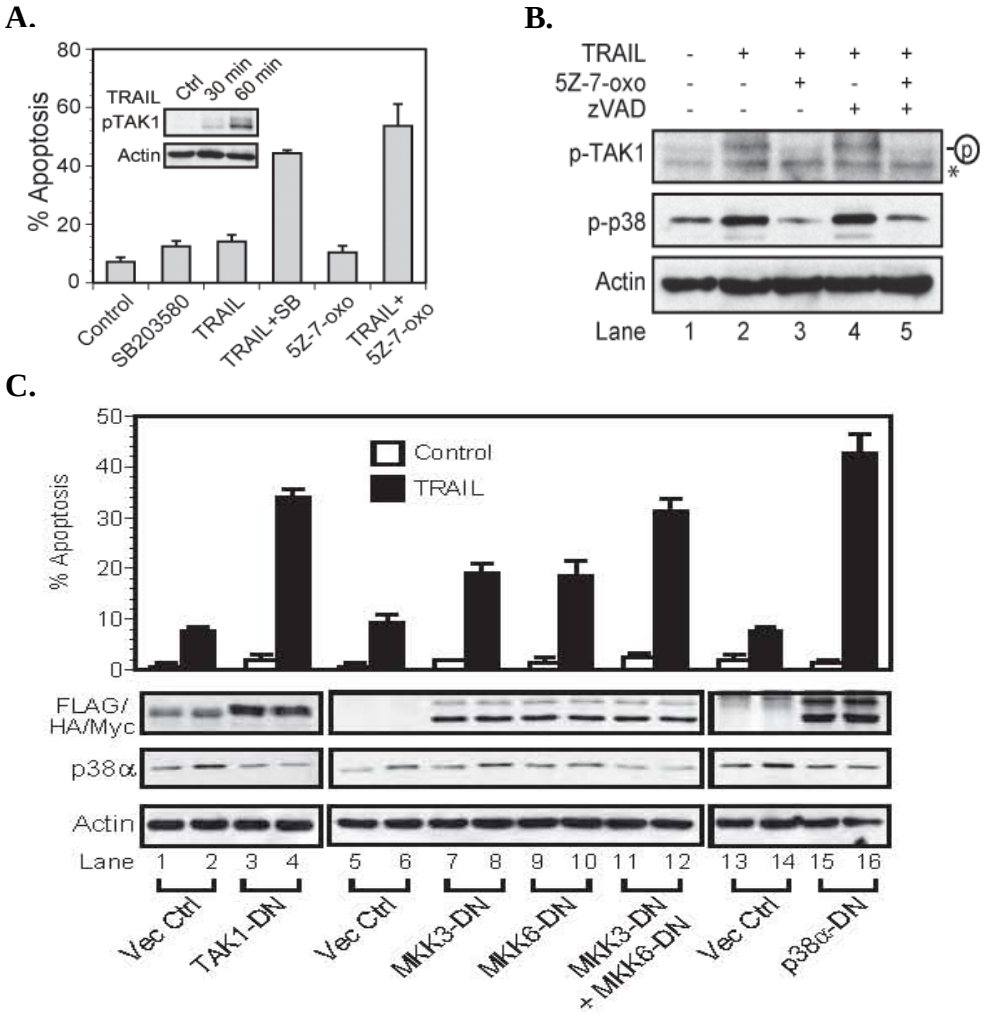


Figure 3.3 TRAIL activates a TAK1→MKK3/6→p38 MAPK pathway that suppresses apoptosis.

(*A and B*) Cells were cotreated with TRAIL ± 5Z-7-oxozeaenol (1 μM) ± zVAD-fmk (50 μM) and were blotted for phosphorylated p-TAK1 and p-p38. In addition, cells were treated with TRAIL ± 5Z-7-oxozeaenol or SB203580 and were assayed for cell death by Annexin V/PI staining and flow cytometry. (*C*) DU145 cells were transiently transfected with pEGFP for 24 h, along with empty vectors, or those expressing dominant-negative mutants of TAK1, MKK3, MKK6, or p38α. Cells were then exposed to TRAIL for 8 h, stained with Hoechst 33258, and assayed for apoptosis by measuring the percentage of GFP+cells with condensed nuclei. Cell lysates were also immunoblotted for FLAG-TAK1-DN, HA-MKK3-DN, HA-MKK6-DN, Myc-p38-DN, and endogenous p-p38α.

TRAIL-mediated activation of p38α. Exposure of DU145 cells to TRAIL resulted in the phosphorylation/activation of TAK1 within ~30-60 min, prior to (or concomitant with) activation of p38α (Fig. 3.1A; Fig. 3.3A, inset), and did so in a caspase-independent manner (Fig. 3.3B, lanes 1, 2 and 4), whereas ASK1 failed to undergo phosphorylation at Thr-845 (data not shown). 5Z-7-oxozeaenol, a resorcylic lactone of fungal origin, has been reported to selectively inhibit TAK1 (Ninomiya-Tsuji et al., 2003; Yao et al., 2007). Therefore, we pretreated cells with 5Z-7-oxozeaenol and found that it inhibited TRAIL-induced activation of TAK1 and p38α (Fig. 3.3B, lanes 1-3 and 5), and correspondingly, potentiated TRAIL-induced apoptosis at levels comparable to TRAIL plus SB203580 (Fig. 3.3A). Finally, to confirm our results with 5Z-7-oxozeaenol, we determined the effects of dominant-negative TAK1 (K63A) and p38α (D168A) mutants on TRAIL-induced activation of p38α and apoptosis in DU145 cells (Fig. 3.3C). Dominant-negative MKK3 (S189A/T193A) and MKK6 (S207A/T211A) mutants were also examined, as these two MKKs play redundant but essential roles in TNF-dependent activation of p38 MAPKs (Brancho et al., 2003). As expected, disruption of the TAK1 $\rightarrow$ MKK3/MKK6 $\rightarrow$ p38 MAPK pathway, at any step, suppressed the phosphorylation/activation of p38α and resulted in significant sensitization of cells to TRAIL-induced cell death (Fig. 3.3C).

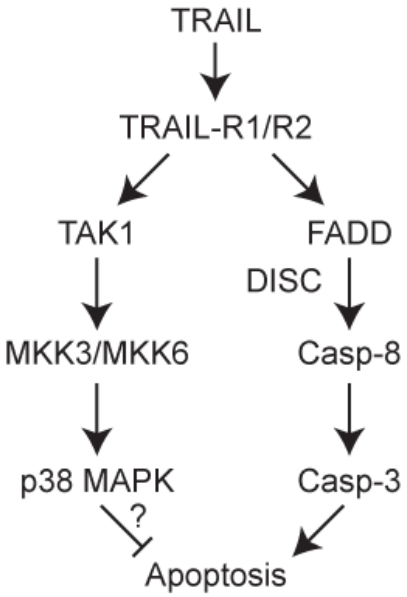


Figure 3.4 TRAIL mediates the activation of both a prosurvival and prodeath pathways.

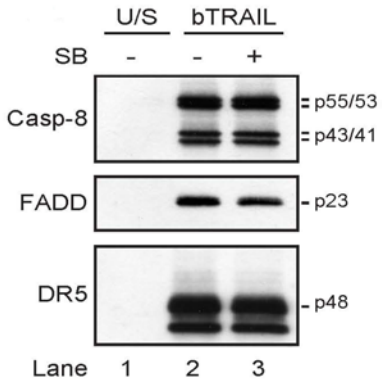
Scheme of the antiapoptotic TAK1→MKK3/MKK6→p38 MAPK and proapoptotic FADD→caspase-8→caspase-3 pathways initiated by TRAIL.

3.2.2 p38 MAPKs suppress TRAIL-induced apoptosis downstream of the DISC by inhibiting MOMP and the release of Smac/DIABLO

Our initial results suggested a simple model, wherein TRAIL receptor stimulation resulted in the activation of a prosurvival TAK1 → MKK3/MKK6 → p38 MAPK pathway and a prodeath TRAIL-R1/TRAIL-R2 → FADD → caspase-8 pathway (Fig. 3.4). It remained unclear, however, how p38α activation prevented apoptosis. To address this question, we first analyzed the effects of SB203580 on formation of the TRAIL DISC. As shown in Fig. 3.5A, SB203580 had no effect on the recruitment of the adapter protein FADD to TRAIL-R2, or the subsequent recruitment and activation of the initiator procaspase-8 (Fig. 3.5A). FLIP overexpression, which can inhibit the activation of procaspase-8 within the DISC and is often cited as a cause of TRAIL resistance, was likewise unaffected by SB203580 (Fig. 3.5B). In contrast, an examination of mitochondria revealed that inhibition of p38 MAPKs was required for TRAIL-induced MOMP, as determined by the mitochondrial release of cytochrome c and Smac/DIABLO into the cytosol (Fig. 3.6).

Cytochrome c induces formation of the apoptosome and sequential activation of caspases-9 and -3 (Bratton et al., 2000). However, in the case of death receptor stimulation, it is often the release of Smac/DIABLO, rather than

A.



B.

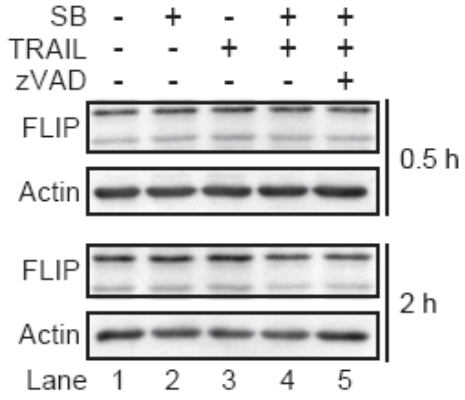


Figure 3.5 p38 MAPKs have no effect on TRAIL DISC formation or basal FLIP expression.

(*A*) DU145 cells were treated with biotinylated-TRAIL (bTRAIL; 500 ng/mL) ± SB203580 (50 μM), and DISC analyses were performed as described in the methods. Note, the unstimulated control (U/S) was obtained by adding bTRAIL to lysed control cells to rule out nonspecific ligand interactions. (*B*) Cells were treated with TRAIL ± SB203580, and immunoblotted for FLIP.

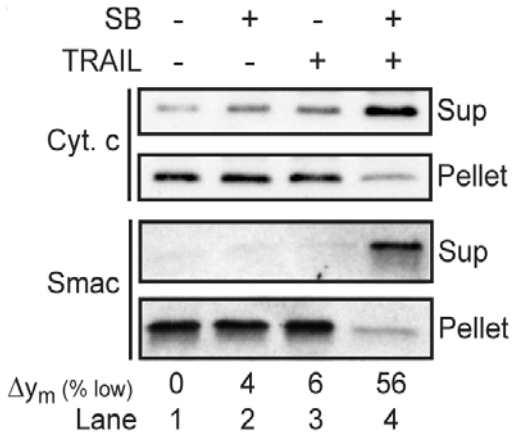


Figure 3.6 p38 MAPKs inhibit MOMP.

Cells were treated with TRAIL ± SB203580, and mitochondrial pellets or supernatants were immunoblotted for cytochrome c or Smac/DIABLO.

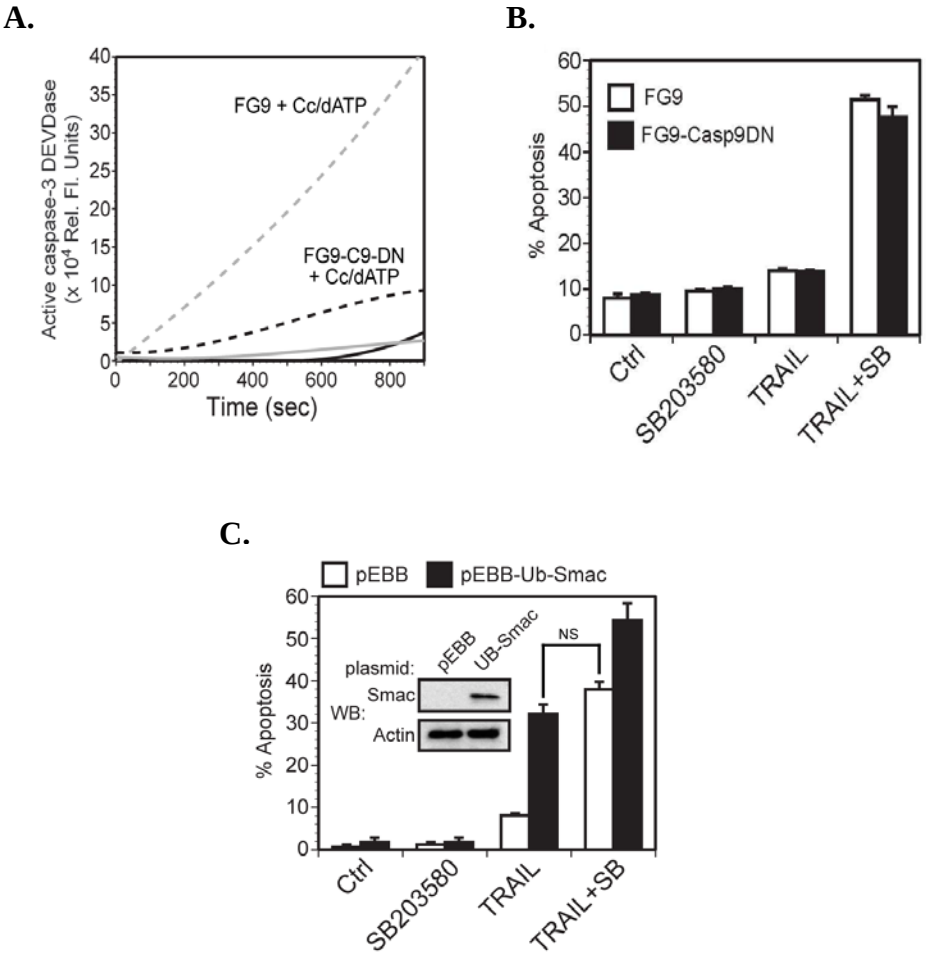


Figure 3.7 The release of Smac/DIABLO, rather than cytochrome c, is essential for TRAIL-induced apoptosis.

(*A and B*) DU145 cells were infected with empty virus or virus expressing a dominant-negative caspase-9, treated with TRAIL ± SB203580, and assayed for cell death by Annexin V/PI staining and flow cytometry. Each bar represents the mean of three separate experiments ± SEM. To confirm the effectiveness of the dominant-negative caspase-9, naive lysates from infected cells were incubated with cytochrome c and dATP and assayed for caspase-3/-7 DEVDase activity. (*C*) DU145 cells were transiently transfected with pEGFP for 24 h, along with pEBB-ubiquitin or pEBB-ubiquitin-Smac/DIABLO, and the cells were then exposed to TRAIL ± SB203580 for 8 h, stained with Hoechst 33258, and assayed for the percentage of GFP+ apoptotic cells. Each bar represents the mean of three separate experiments ± SEM. *NS*, denotes a lack of statistical significance.

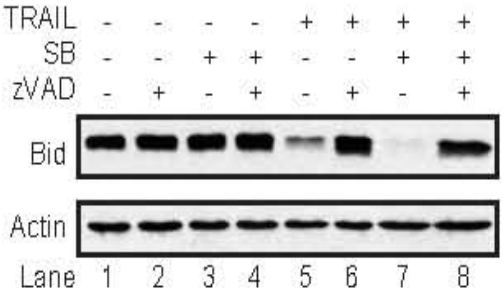
cytochrome c, that is critical for cell killing. In these instances, active caspase-8 processes procaspase-3, but caspase-3 is inhibited by IAPs, and cytosolic Smac/DIABLO is required to antagonize IAPs and relieve the inhibition of caspase-3 (Bratton and Cohen, 2003). We therefore stably infected cells with a lentivirus expressing dominant-negative caspase-9 (C287A), a potent inhibitor of the apoptosome (Choi et al., 2009), or transiently transfected cells with a ubiquitin-Smac/DIABLO fusion construct that produces mature cytosolic Smac/DIABLO (Hunter et al., 2003). To confirm the effectiveness of the dominant-negative caspase-9, lysates from untreated cells were assayed for apoptosome activity in response to the addition of cytochrome c and dATP. Overexpression of dominant-negative caspase-9 had little effect on TRAIL/SB203580-induced apoptosis in DU145 cells (Fig. 3.7B), even though it clearly inhibited the activation of endogenous caspase-9 in cytochrome c/dATP-activated lysates (Fig. 3.7A). Smac/DIABLO, on the other hand, dramatically potentiated TRAIL-induced cell death, even in the absence of SB203580 (Fig. 3.7C). Thus, the TAK1 $\rightarrow$ MKK3/MKK6 $\rightarrow$ p38 MAPK pathway appeared to suppress TRAIL-induced apoptosis by preventing MOMP and the release of Smac/DIABLO.

3.2.3 p38 MAPKs inhibit TRAIL-induced MOMP, in spite of BID activation, through transcriptional upregulation of *MCL-1*

Death receptors normally engage the mitochondrial pathway through caspase-8-dependent cleavage and activation of the proapoptotic BH3-only protein, BID (Chai et al., 2000; Li et al., 1998; Luo et al., 1998). Though controversial, truncated BID (tBID) and other BH3-only proteins then directly activate the multidomain proapoptotic BCL-2 family members, BAX or BAK (*direct activation model*), and/or antagonize the antiapoptotic Bcl-2 family members, BCL-2, BCL-XL, or MCL-1, thereby relieving their inhibition of BAX and BAK (*indirect activation model*) (Youle and Strasser, 2008). We treated DU145 cells with TRAIL ± SB203580 and examined them for BID cleavage and downstream BAK activation. Remarkably, exposure to TRAIL alone induced near complete cleavage of BID (Fig. 3.8A, lane 5), but in the absence of SB203580, tBID failed to induce the conformational change in BAK required for its activation (Fig. 3.8B). Thus, consistent with our previous DISC results, TRAIL receptor stimulation induced significant caspase-8 activation and BID cleavage, but tBID was unable to induce BAK oligomerization and MOMP unless the antiapoptotic p38 MAPK pathway was disrupted (Fig. 3.5A; Fig. 3.6; Fig. 3.8A, B).

Given the aforementioned results, we considered that p38 MAPKs might regulate the expression of BCL-2, BCL-x_L, or MCL-1. Indeed, following treatment with SB203580, basal MCL-1 (but not BCL-2 or BCL-x_L) expression levels were reduced, both at the protein and transcriptional levels (Fig. 3.9A, lanes

A.



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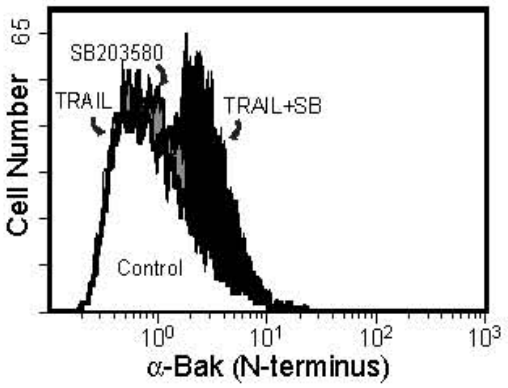


Figure 3.8 Inhibition of p38 MAPKs is required for MOMP, in spite of Bid activation.

(*A*) DU145 cells were treated with TRAIL (500 ng/mL) ± SB203580 (50 μM) ± zVAD-fmk (50 μM), immunoblotted for BID cleavage, and *(B)* assayed for BAK activation by flow cytometry using an epitope-specific antibody that recognizes its active conformation.

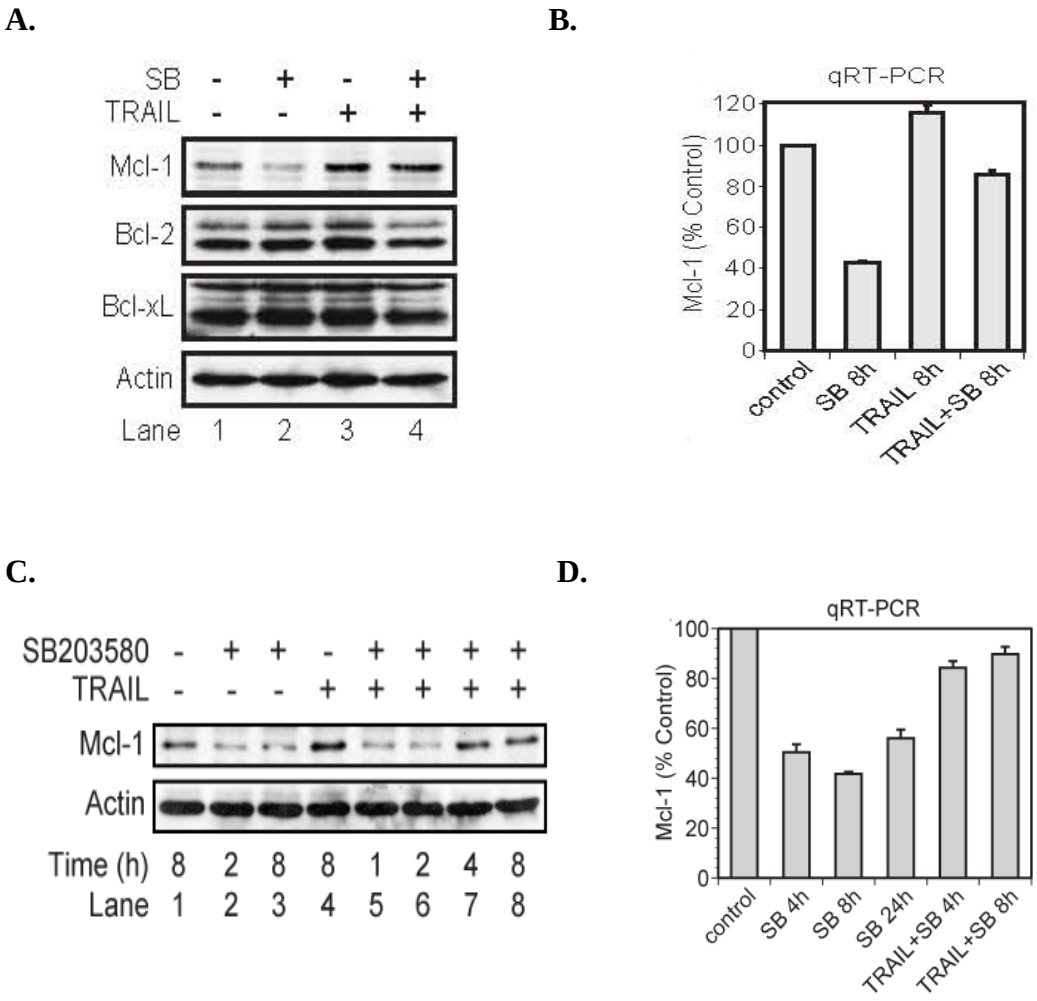


Figure 3.9 p38 MAPKs regulate the transcription of MCL-1.

(*A and B*) Cells were treated with TRAIL ± SB203580 and assayed for expression of antiapoptotic multidomain BCL-2 family members, MCL-1, BCL-2, and BCL-X_L, at the protein (immunoblotting) and/or transcriptional (qRT-PCR) levels. (*C and D*) Time-course experiments were performed (as described above) to assess temporal changes in MCL-1 expression following treatment with TRAIL ± SB203580.

1 and 2; Fig. 3.9B), whereas TRAIL treatment alone led to an increase in MCL-1 expression (Fig. 3.9A, lanes 1 and 3; Fig. 3.9B). Surprisingly, when used in combination, MCL-1 levels were largely maintained, but it was important to note that these assays were performed at 8 h, when the combination of TRAIL plus SB203580 induced significant apoptosis (Fig. 3.9A, lane 4; Fig. 3.9B). Subsequent time-course experiments revealed that SB203580 suppressed the basal and upregulated expression of MCL-1 by TRAIL during the first 2-3 h of treatment and that MCL-1 levels did not rebound until later ($\geq$ 4 h) after the activation of caspases (Fig. 3.9C, lanes 4-8; Fig. 3.9D). Thus, in the presence of SB203580, cells were sensitized to TRAIL for at least 3-4 h, although recovery of Mcl-1 levels by 4 h after TRAIL treatment may have protected some cells from death. To confirm that the loss of MCL-1 expression was due to a decrease in transcription and was not the result of an increase in protein turnover, we transiently expressed MCL-1 from a CMV promoter and followed the turnover of MCL-1 in the presence of cycloheximide, an inhibitor of protein synthesis. Since ERK-dependent phosphorylation of MCL-1 at Thr-163 has been shown to regulate the turnover of MCL-1 (Domina et al., 2004), we compared the effects of SB203580 with the ERK inhibitor, U0126. As shown, in Fig. 3.10, while treatment with U0126 led to rapid turnover of MCL-1 (*lower panels*), inhibition of p38 MAPKs with SB203580 had no such effect (*upper panels*). Thus,

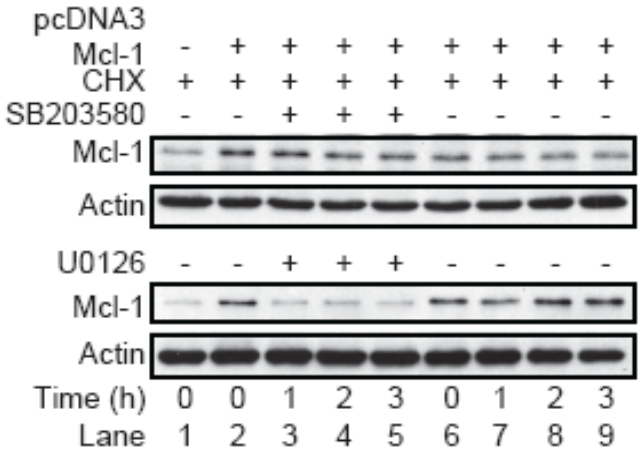


Figure 3.10 p38 MAPKs have no effect on MCL-1 turnover.

(*G*) DU145 cells were transiently transfected with pcDNA3-MCL-1 for 24 h, after which cycloheximide (CHX; 1μM) was added, along with either SB203580 (50 μM) or the ERK inhibitor U0126 (10 μM). Turnover was then assessed by immunoblotting MCL-1 over a 3 h time-course.

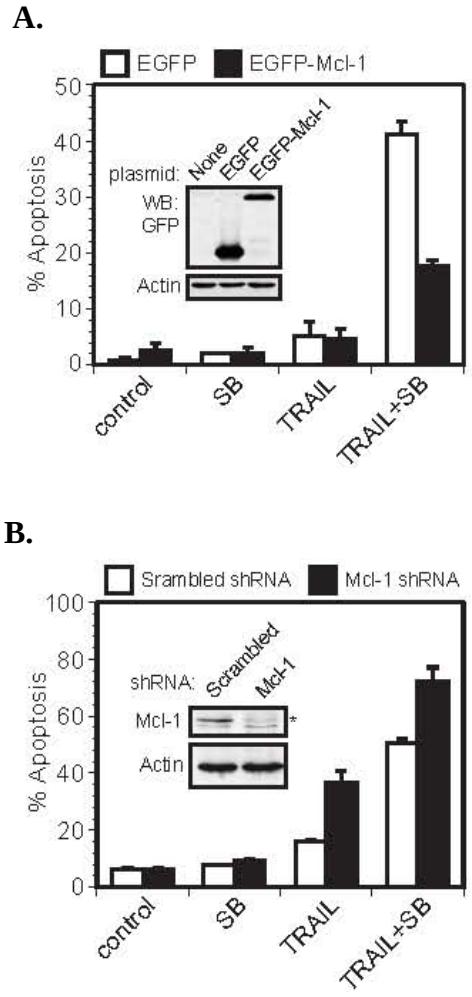
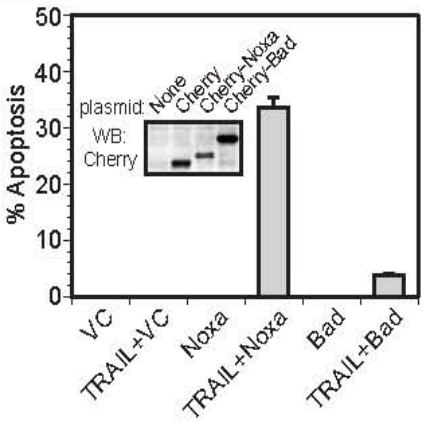


Figure 3.11 MCL-1 mediates TRAIL resistance.

(*A*) DU145 cells were transiently transfected with pEGFP or pEGFP-MCL-1 for 24 h (*see* inset for immunoblot of expressed proteins), exposed to TRAIL ± SB203580 for 8 h, stained with Hoechst 33258, and assayed for the percentage of GFP+ apoptotic cells. Each bar represents the mean of three separate experiments ± SEM. (*B*) DU145 cells were transfected with pSuper-scramble or pSuper-MCL-1 to stably knockdown the expression of MCL-1 by RNA interference (*see* inset for knockdown of MCL-1). Cells were then exposed to TRAIL ± SB203580 and assayed for cell death by Annexin V/PI staining and flow cytometry. Each bar represents the mean of three separate experiments ± SEM.

A.



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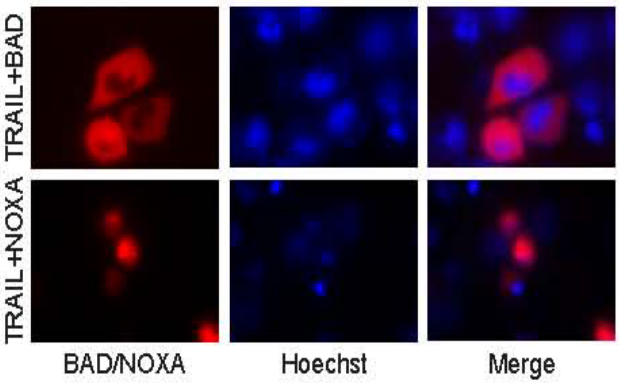

Figure 3.12 Overexpression of NOXA potentiates TRAIL-induced apoptosis.

(*A and B*) DU145 cells were transiently transfected with pmCherry, pmCherry-NOXA, or pmCherry-BAD for 24 h (*see* inset for immunoblot of expressed proteins), exposed to TRAIL ± SB203580 for 8 h, stained with Hoechst 33258, and assayed for the percentage of mCherry + apoptotic cells. Each bar represents the mean of three separate experiments ± SEM.

collectively, the data argued strongly that p38 MAPK activity regulated the expression of MCL-1 at the transcriptional level.

We next confirmed the importance of MCL-1 for mediating TRAIL resistance using several approaches. Firstly, we found that ectopic expression of EGFP-MCL-1 significantly inhibited cell death induced by TRAIL plus SB203580 (Fig. 3.11A), whereas selective downregulation of endogenous MCL-1 by RNA interference sensitized cells to TRAIL alone, at a level comparable to scrambled control cells treated with TRAIL plus SB203580 (Fig. 3.11B). Secondly, since the BH3-only proteins, NOXA and BAD, selectively antagonize MCL-1 and BCL-2/BCL-XL/BCL-W, respectively (Chen et al., 2005b), we expressed NOXA or BAD as Cherry fusion proteins and found that only NOXA sensitized DU145 cells to TRAIL (Fig. 3.12A, B). Thus, while TRAIL receptor stimulation led to near complete cleavage of BID, the intracellular concentration of tBID was apparently insufficient to fully antagonize MCL-1 and alleviate the inhibition of BAK by MCL-1; or alternatively, MCL-1 sequestered all of the available tBID and prevented its activation of BAK. Consistent with either interpretation, ectopic overexpression of tBID-EGFP induced significant apoptosis, but when combined with SB203580 (which inhibited basal p38 activity and downregulated basal MCL-1 levels), ~90% of the cells underwent apoptosis (Fig. 3.13). Thus, following TRAIL receptor stimulation, disruption of the TAK1

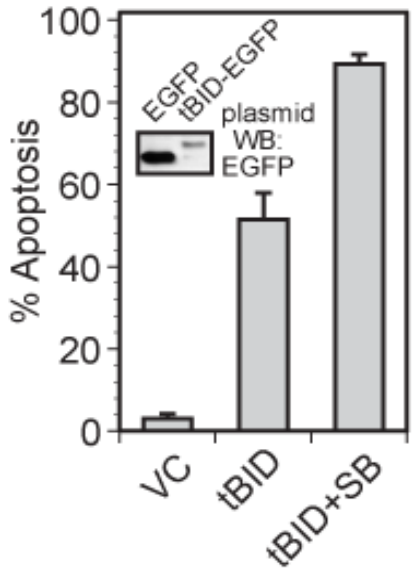

Figure 3.13 SB203580 sensitizes cells to tBid-induced apoptosis.

DU145 cells were transiently transfected with EGFP or tBID-EGFP for 24 h (*see* inset for immunoblot of expressed proteins), exposed to DMSO or SB203580 for 8 h, stained with Hoechst 33258, and assayed for the percentage of GFP+ apoptotic cells. Each bar represents the mean of three separate experiments ± SEM.

→ MKK3/MKK6 → p38 MAPK pathway was required to downregulate MCL-1 and facilitate tBID-induced BAK activation, MOMP, and cell death.

3.3 Discussion

TNF receptor stimulation activates various antiapoptotic signaling pathways that typically subvert an otherwise proapoptotic extrinsic pathway, resulting in minimal (if any) death in most cell-types (Wajant et al., 2003). TRAIL receptor stimulation, by comparison, induces apoptosis selectively in tumor cells, but unfortunately ~50% of tumors possess or acquire resistance to TRAIL. Previous studies have shown that TRAIL, like TNF, activates ERK, JNK, and p38 kinases, but the magnitude of the responses is generally modest, and it remains unclear if these pathways play significant roles in mediating TRAIL resistance. In this study, we have found that stimulation of TRAIL receptors activates p38α that is responsible for resistance to apoptosis (Fig 3.1; Fig. 3.2). We next questioned how p38α was activated through TRAIL. Since TNF reportedly activates p38α through the activation of MLKs, TAK1 or ASK1 (Cheung et al., 2003; Ichijo et al., 1997a; Tobiume et al., 2001; Wang et al., 2001) we sought to determine if either of these MKKKs was essential for TRAIL-mediated p38α activation. We have observed that TAK1 → MKK3/MKK6 → p38 MAPK pathway that suppresses apoptosis is activated upon TRAIL stimulation (Fig. 3.3). Taken together, our data suggest that TRAIL receptor

stimulation leads to the activation of an antiapoptotic TAK1 → MKK3/MKK6 → p38 MAPK pathway and a proapoptotic TRAIL-R1/TRAIL-R2 → FADD → caspase-8 pathway, and the antiapoptotic pathway induced by TRAIL inhibits the TRAIL-activated proapoptotic pathway.

The mechanism by which p38 MAPKs prevented apoptosis in DU145 prostate cancer cells, however, remained unclear. To address this question, we tested the effect of p38 MAPKs inhibition on TRAIL DISC formation and the expression of FLIP, which is often cited as a cause of TRAIL resistance. No effects were observed (Fig. 3.5). However, an examination of mitochondria revealed that inhibition of p38 MAPKs was essential for TRAIL-induced MOMP (Fig. 3.6). Indeed, consistent with previous studies in other cancer cell types, from our laboratory and others, we found that release of Smac (rather than cytochrome c) was essential for TRAIL-induced apoptosis in DU145 prostate cancer cells (Fig. 3.7). Taken together, our data indicate that the TRAIL-activated p38 MAPK pathway results in inhibition of TRAIL-induced apoptotic pathway by preventing MOMP. Since MOMP is highly controlled by BCL-2 family members, we speculated that p38 MAPKs might regulate BCL-2 family members and found that p38 MAPKs regulates transcription of MCL-1 (Fig. 3.9) and that MCL-1 is critical for mediating TRAIL resistance (Fig. 3.11; Fig. 3.12).

Exposure to TRAIL alone led to robust DISC formation, caspase-8 activation, and near complete BID cleavage, ruling out a prominent role for c-

FLIP in mediating TRAIL resistance (Fig. 3.5; Fig. 3.8). Endogenous tBID, however, did not induce apoptosis unless MCL-1 expression levels were downregulated through simultaneous inhibition of the TRAIL-activated p38 pathway, knockdown of MCL-1 by RNA interference, or antagonism of MCL-1 by ectopically-expressed NOXA (Fig. 3.11; Fig. 3.12). The fact that ectopic overexpression of tBID could induce apoptosis, whereas NOXA could not, suggests that there was simply insufficient generation of endogenous tBID following TRAIL treatment to fully antagonize MCL-1 and/or directly activate BAK. Our data do not exclude either the *direct* or *indirect activation models* as previously posited, but it is worth noting that BIM (which like tBID is proposed to be a "direct activator") has been shown to antagonize antiapoptotic BCL-2 family members and directly activate BAX (Merino et al., 2009).

An interesting observation we made while investigating the role of p38 MAPKs in the regulation of MCL-1 was that low MCL-1 levels reduced by SB203580 treatment rebounded upon cotreatment with TRAIL. This observation led us to speculate that a secondary pathway must exist to upregulate the expression of MCL-1 following cotreatment with TRAIL plus SB203580.

Chapter 4. TRAIL-induced MOMP activates a ROS-dependent JNK pathway that upregulates MCL-1 expression and partially rescues cells from apoptosis

4.1 Introduction

MCL-1 is a highly expressed prosurvival protein in human malignancies and its cellular expression is tightly regulated *via* multiple mechanisms (Akgul, 2009). Regulation of MCL-1 expression occurs at multiple levels. Furthermore, it is rapidly downregulated during apoptosis, whereas its expression is induced by survival stimuli. Several kinases such as MAPKs, Janus kinases (JAKs), AKT, and glycogen synthase kinase (GSK)-3ß have been shown to phosphorylate MCL-1 leading to regulation of MCL-1 expression at multiple levels (Akgul, 2009; Craig, 2002). The rapid and complex regulation allows MCL-1 to be adapted in response to rapidly changing conditions.

Reactive oxygen species (ROS) have been implicated in a wide variety of pathologies, including cancer, type II diabetes, arteriosclerosis, chronic inflammatory processes, ischemia/reperfusion injury, and various neurodegenerative diseases (Droge, 2002). The mitochondrial respiratory chain is the major source of intracellular ROS generation. Cellular metabolism depends on the continuous supply of ATP from the mitochondria. Thus, any damage that impairs the function of the respiratory chain might have an impact on cell

viability. To protect cells from oxidative insult, mitochondria contain an elaborate defense system to detoxify ROS and repair ROS-induced damage.

Oxidative stress is generally defined as an imbalance that favors the production of ROS over antioxidant defenses. About 1-2% of the molecular oxygen consumed during normal physiological respiration is converted into superoxide radicals. The one electron reduction of molecular oxygen produces a relatively stable intermediate, superoxide anion ($O_2^{\cdot -}$), which serves as the precursor of most ROS and the dismutation of superoxide anions by superoxide dismutases results in the production of hydrogen peroxide (H_2O_2). Fe^{2+}- (or Cu^{2+})-driven cleavage of H_2O_2 in a Fenton reaction can generate highly reactive hydroxyl radical ($OH^{\cdot}$) (Klaunig and Kamendulis, 2004). The mitochondrial electron transport chain contains several redox centers that leak electrons to molecular oxygen, serving as a primary source of superoxide production. $O_2^{\cdot -}$ production by complex I has been shown to be stimulated in the presence of succinate, a substrate of complex II (Liu et al., 2002) and is generated on the matrix side of IMM (de Vries, 1986). In addition to complex I, complex III is an important site of $O_2^{\cdot -}$ production (Grigolava et al., 1980), especially when mitochondrial respiration is suppressed by antimycin A, an inhibitor of complex III, $O_2^{\cdot -}$ produced at this site appears on both sides of the IMM (Muller et al., 2004).

ROS have been reported to affect the apoptotic process in the mitochondria. For instance, cytochrome c, one of the critical players within the electron transport chain, is normally bound to the IMM by an association with the anionic phospholipid cardiolipin. ROS facilitate the detachment of cytochrome c from cardiolipin *via* oxidation of cardiolipin, leading to a reduction in its affinity for cytochrome c. This results in an increase in the free pool of cytochrome c within the inner membrane space, which can in turn be released into the cytosol upon MOMP (Kagan et al., 2005).

In the present study, I have investigated the role of a secondary ROS-dependent JNK pathway activated by MOMP in the regulation of MCL-1 expression.

4.2 Results

As already noted, SB203580 inhibited TRAIL-activated p38 MAPKs, thereby preventing the expression of MCL-1 during the first 2-3 h following treatment with TRAIL and sensitized the cells to apoptosis (Fig. 3.1; Fig. 3.2; Fig. 3.9C). MCL-1 expression levels, however, partially rebounded ≥4 h later, concomitant with caspase activation (Fig. 3.9C, D; data not shown). MCL-1 expression levels are tightly regulated by phosphorylation from several kinases. Thus, we speculated that, in addition to transcriptional regulation of MCL-1 by p38 MAPKs, MCL-1 expression levels might also regulated by another pathway

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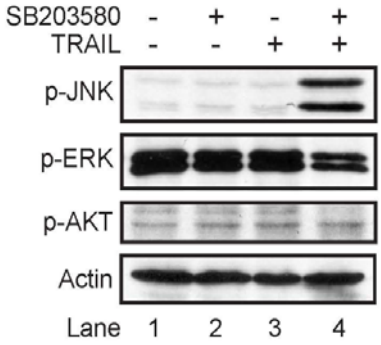

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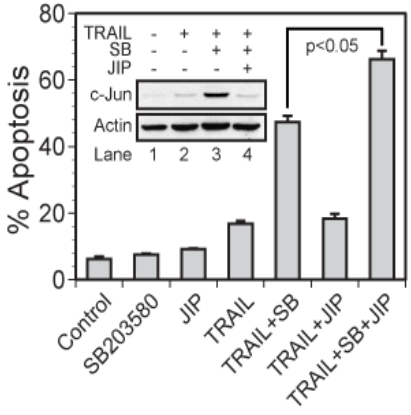
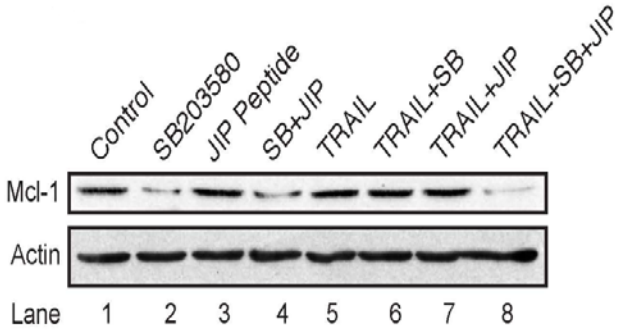


Figure 4.1 JNKs activated by cotreatment with TRAIL plus SB203580 partially protects cells from apoptosis.

(*A*) DU145 cells were treated with TRAIL (500 ng/mL) ± SB203580 (50 μM) and immunoblotted for active p-JNK (Thr-183/Tyr-185), p-ERK (Thr-202/Tyr-204), and p-AKT (Ser-473). (*B*) Cells were treated with TRAIL ± SB203580 ± JIP peptide (10 μM) and the percentage of cell death was determined by Annexin V/PI staining and flow cytometry. Each bar represents the mean of three separate experiments ± SEM. Note, in the inset to panel B, the inhibitory effect of JIP was confirmed, as it inhibited JNK-dependent phosphorylation of c-Jun.

A.



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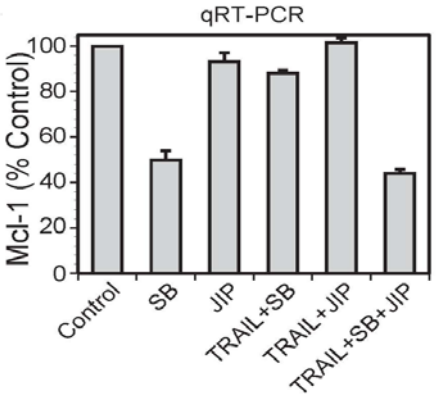


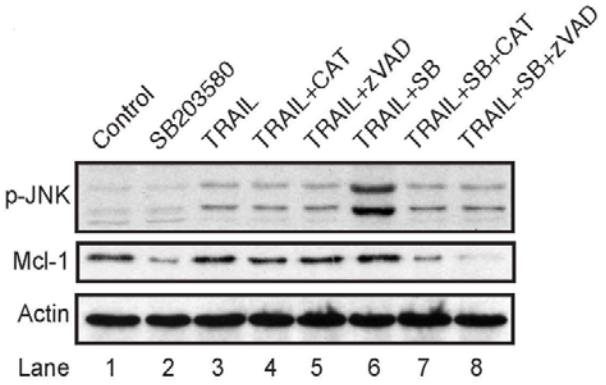
Figure 4.2 TRAIL/SB20380-activated JNKs transcriptionally upregulate MCL-1 expression.

(*A*) Cells were treated with TRAIL ± SB203580 ± JIP peptide (10 μM) and assayed for MCL-1 expression at the protein (immunoblotting) and (*B*) transcriptional (qRT-PCR) levels. Each bar represents the mean of three separate experiments ± SEM.

upon cotreatment with TRAIL plus SB203580. In order to characterize this resurgence in MCL-1 expression and to assess its potential impact on cell death, we treated cells with TRAIL ± SB203580 and once again assayed them for the activation of several kinases, including JNKs, ERKs, and AKT. Interestingly, ERKs were constitutively active, and there was no difference in AKT phosphorylation, whereas JNKs were robustly activated, but only in cells cotreated with TRAIL plus SB203580 (Fig. 4.1A, lane 4). More importantly, a cell-permeable peptide inhibitor of JNK, derived from JNK interaction protein (JIP), inhibited JNK activity (Fig. 4.1B, inset, lanes 3 and 4), prevented entirely the rebound in MCL-1 protein expression levels (Fig. 4.2A, lanes 6 and 8), and further sensitized cells to apoptosis (Fig. 4.1B; compare TRAIL+SB+JIP with TRAIL+SB, $p<0.05$). Consistent with the result shown in Fig. 4.2A, the rebound in MCL-1 mRNA levels were completely blocked in the presence of JIP.

Since JNKs were activated relatively late following cotreatment with TRAIL plus SB203580, we questioned whether caspases might play a role in their activation, and indeed, addition of zVAD-fmk inhibited fully the activation of JNKs (Fig. 4.3A, lanes 6 and 8). Given that (*i*) caspase-dependent cleavage of BID and downregulation of MCL-1 was required to induce MOMP following exposure to TRAIL plus SB203580 (Fig. 3.8, 13), (*ii*) mitochondrial perturbation often leads to the production of ROS (Ricci et al., 2004), and (*iii*) ROS are implicated in the TNF-dependent activation of JNK *via* activation of ASK1 and/or

A.



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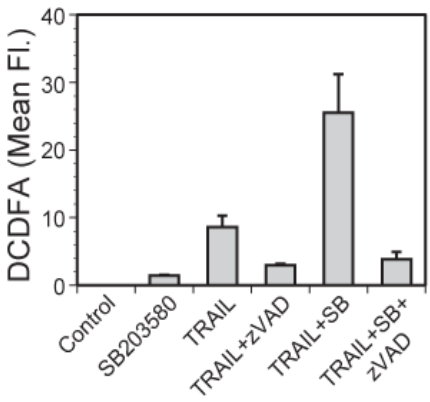

Figure 4.3 JNK activation by TRAIL plus SB203580 is ROS dependent.

(*A*) DU145 cells were treated with TRAIL ± SB203580 ± the ROS scavenger catalase (CAT; 100 U) or the polycaspase inhibitor zVAD-fmk (50 μM), and immunoblotted for MCL-1 expression, and (*B*) assayed for ROS production by flow cytometry using the fluorescent dye DCDFA. Each bar represents the mean of three separate experiments ± SEM.

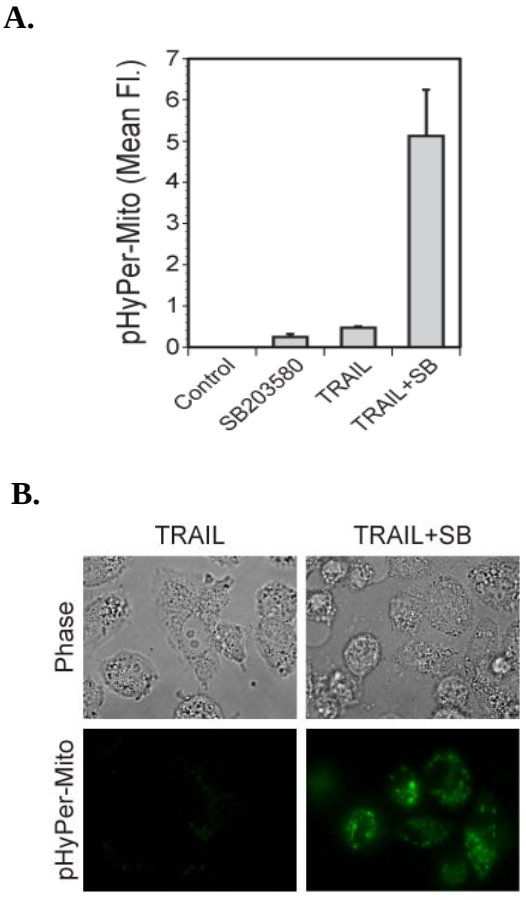

Figure 4.4 TRAIL-induced MOMP activates the ROS-dependent JNK pathway.

(*A*) DU145 cells were treated with TRAIL ± SB203580 and assayed for ROS production by flow cytometry using pHyPer-Mito, a mitochondrial-localized oxidant-activated fluorescent protein and (*B*) images were captured by fluorescence microscopy.

inhibition of MKPs (Kamata et al., 2005; Tobiume et al., 2001), we further speculated that ROS might be responsible for the activation of JNKs in our studies. As predicted, TRAIL plus SB203580 induced a significant caspase-dependent increase in the production of ROS, as determined by the oxidant-sensitive fluorescent dye DCDFA (Fig. 4.3B), and addition of the ROS scavenger catalase prevented the activation of JNKs (Fig. 4.3A, lanes 6 and 7). DCDFA however is largely cytosolic, so to specifically invoke a role for mitochondria in the production of ROS, we transfected cells with a mitochondrial-localized circularly-permuted yellow fluorescent protein, inserted into the regulatory domain of the oxidant sensitive *E. coli* transcription factor OxyR (pHyPer-Mito) (Belousov et al., 2006). As shown in Fig. 4.4, control cells and those treated with SB203580 or TRAIL alone exhibited little activation of the pHyPer-Mito probe. In stark contrast, cells cotreated with TRAIL plus SB203580 — a combination that induced MOMP, cytochrome c release, and a loss in $\Delta\psi m$ (Fig. 3.6) — displayed a profound increase in mitochondrial fluorescence (Fig. 4.4A, B). Taken together, the data supported a model wherein TRAIL plus SB203580 induced BID activation and downregulation of MCL-1, resulting in MOMP, cytochrome c release, a loss in $\Delta\psi m$, increased ROS production, activation of JNKs, and ultimately, a resurgence in the expression of MCL-1 (Fig. 4.5). Thus, even during times of profound stress, *e.g.* following MOMP, the affected cells initiated a complex secondary survival pathway that converged upon MCL-1,

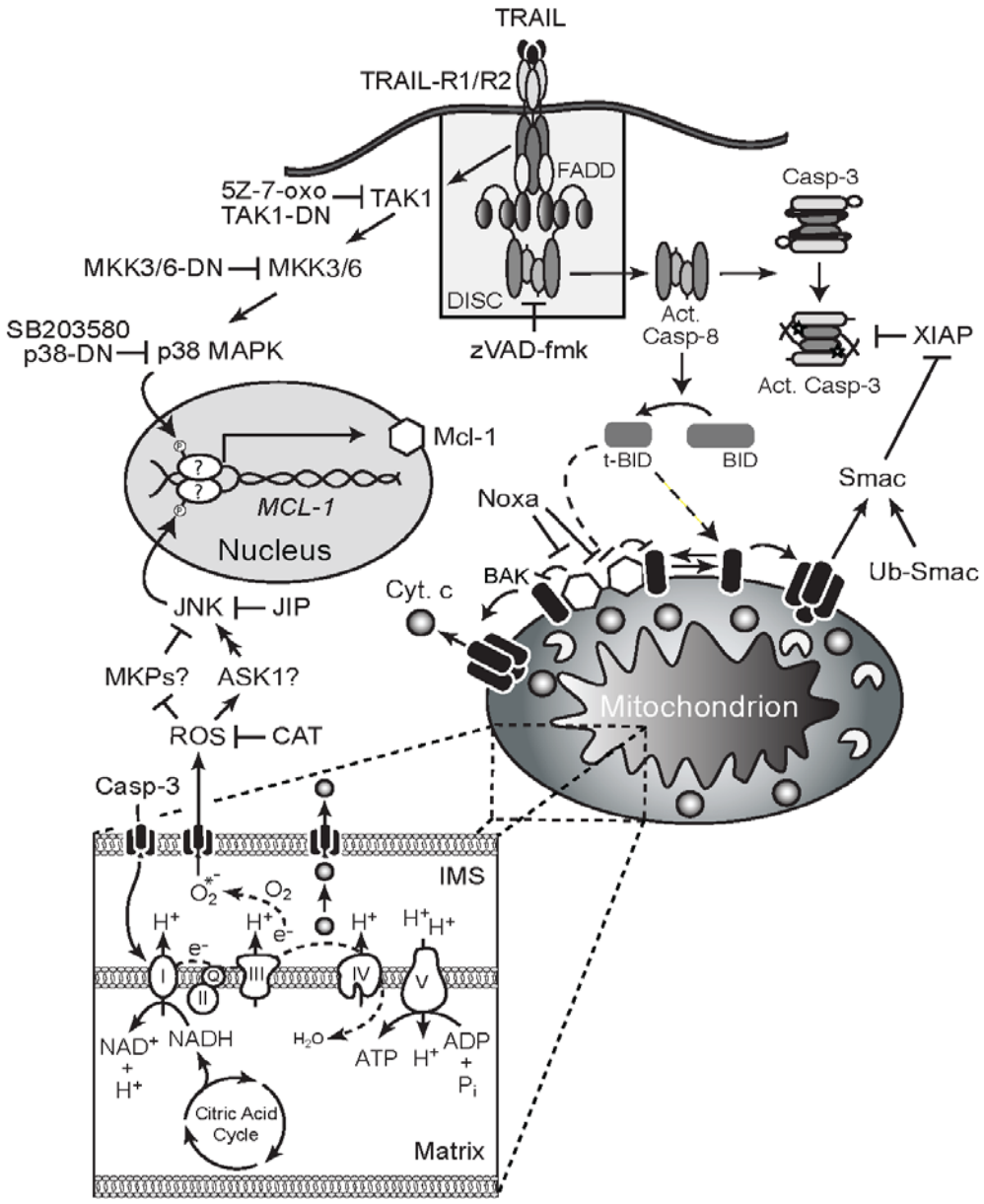

Figure 4.5 Model of TRAIL-activated proapoptotic and antiapoptotic pathways.

reemphasizing the importance of MCL-1 as a regulator of TRAIL-induced cell death.

4.3 Discussion

MCL-1 has been shown to mediate TRAIL resistance in human cancer cells (Han et al., 2006; Meng et al., 2007; Ricci et al., 2007) and consistent with previous studies, our studies in prostate cancer cells reveal the critical importance in maintaining MCL-1 expression levels in order to suppress TRAIL-induced apoptosis. Indeed, even following MOMP, mitochondrial-generated ROS stimulated a secondary JNK pathway that transcriptionally upregulated the expression of MCL-1 and partially rescued some cells from death (Fig. 4.1-4. 4).

The mitochondrial-generated ROS were most likely produced at complex III, due to inefficient electron transfer following the release of cytochrome c, or alternatively at complex I, due to caspase-dependent cleavage of NDUFS1 (Fig. 4.5) (Ricci et al., 2004). Irrespective, these results are in stark contrast to TNF signaling, wherein ROS are produced by NADPH oxidases upstream of mitochondria and in turn activate ASK1 or inactivate MKPs, resulting in prolonged JNK activation, MOMP, and apoptosis (Kamata et al., 2005; Kim et al., 2007; Tobiume et al., 2001). Our results show that p38 MAPK regulates the transcription of MCL-1 (Fig. 3.9) and that JNK also appears to control MCL-1 expression levels *via* transcription following cotreatment with TRAIL plus

SB203580 (Fig. 4.2). However, at this stage, the transcription factor(s) responsible for p38 MAPK and JNK-dependent upregulation of MCL-1, following treatment with TRAIL and TRAIL+SB203580, respectively, remain unknown and are currently under investigation. STAT3 and PU.1 have previously been implicated in MCL-1 expression; however, PU.1 is generally restricted to hematopoietic tissues, and dominant-negative mutants of STAT1, STAT3, and ATF2 (a p38α and JNK-targeted transcription factor) failed to potentiate TRAIL-induced apoptosis (data not shown).

Our data propose a model wherein TRAIL stimulates its receptors, TRAIL-R1 and/or TRAIL-R2, resulting in recruitment of the adapter protein FADD and activation of procaspase-8 within the DISC. Caspase-8 subsequently processes procaspase-3, but active caspase-3 is rapidly inhibited by IAPs, including XIAP. Caspase-8 also cleaves and activates BID, and tBID would normally induce BAK-dependent MOMP and release the IAP antagonist Smac/DIABLO. However, TRAIL receptor stimulation simultaneously activates an antiapoptotic TAK1 → MKK3/MKK6 → p38 MAPK signaling pathway that transcriptionally upregulates MCL-1, which in turn sequesters/neutralizes tBID. Disruption of this signaling pathway at any step sensitizes cells to TRAIL, but remarkably, even following MOMP, mitochondrial-generated ROS activate a secondary JNK pathway that similarly leads to transcriptional upregulation of MCL-1 and partial protection from cell death (Fig. 4.5).

Chapter 5. Concluding remarks

Apoptosis is a tightly controlled physiological process of cell elimination that plays a critical role in development and homeostasis (Kerr et al., 1972). It is important for maintaining appropriate cell numbers in tissues, serving as a balance to cell proliferation. Thus, deregulated apoptosis has been implicated in a variety of diseases. Too much apoptosis results in cell-loss disorders, whereas too little apoptosis can prevent tumor cells from dying, leading to cancer (Fadeel et al., 1999b). Cancer is a leading cause of premature human death and commands considerable research attention. However, despite significant progress in the treatment of cancer over the past four decades, the prognosis for many cancers remains poor. Inherent and acquired resistance of tumor cells to existing chemotherapeutics is one of the principal reasons for this and highlights the importance of devising novel therapeutic approaches.

Multicellular organisms normally eliminate damaged cells most effectively through apoptosis, resulting in death. However, one of the hallmarks of cancer cells is active avoidance of apoptosis, thereby promoting their survival. Consequently, methods of inducing apoptosis have become an important approach in the design of new cancer therapies. Many proapoptotic agents have been developed, but their unacceptable toxicity to normal tissues usually limits their use in the clinic.

TRAIL, as one of the TNF family members, induces apoptosis through engagement of death receptors. It has generated tremendous enthusiasm as a potential tumor-specific cancer therapeutics, because as a stable trimer, it selectively induces apoptosis in many transformed cells, but not in most normal cells (Falschlehner et al., 2009). Early-phase clinical trials have been initiated in cancer patients, testing for safety, pharmacokinetics, and preliminary evidence of anti-tumor activity. Recombinant TRAIL (Genentech and Amgen), and monoclonal antibodies targeting TRAIL-R1, such as mapatumumab (HGS-ERT1; Human Genome Sciences), and TRAIL-R2, such as lexatumumab (HGS-ERT2; Human Genome Sciences), AMG 655 (Amgen) and apomab (Genetech) have been developed and are being tested in human clinical trials.

However, many primary tumors are inherently resistant to TRAIL-induced apoptosis and require additional sensitization. Therefore, understanding the mechanisms of TRAIL resistance and developing strategies to overcome that resistance is critical for future success. Many studies over the last decade have proposed mechanisms responsible for TRAIL resistance. However, these mechanisms may differ depending upon the cell-type, etc. Herein, we have demonstrated that TRAIL activates a TAK1 $\rightarrow$ MKK3/MKK6 $\rightarrow$ p38 pathway in prostate cancer cells that transcriptionally upregulates expression of the antiapoptotic BCL-2 family member MCL-1, resulting in TRAIL resistance. TRAIL triggers a prodeath TRAIL-R1/TRAIL-R2 $\rightarrow$ FADD $\rightarrow$ caspase-8

pathway; however, the activity of caspase-3 is largely blocked by IAPs, such as XIAP. Therefore, disruption of the p38 MAPK pathway is required to suppress MCL-1 expression, thereby allowing tBID to activate the proapoptotic BCL-2 family member BAK and stimulate MOMP. This results in release of the IAP antagonist Smac/DIABLO, which relieves the inhibition of caspase-3 by XIAP, leading to apoptosis.

Although MCL-1 is an antiapoptotic BCL-2 family protein that plays essential roles in the development and differentiation of normal cells, deregulation of MCL-1 contributes to human malignancies. Overexpression of MCL-1 has been shown in a variety of human hematopoietic, lymphoid cancers and solid tumors (Aichberger et al., 2005; Derenne et al., 2002; Sieghart et al., 2006), and also appears to be a key factor in the resistance of some cancer types to conventional cancer therapies (Hussain et al., 2007; Nguyen et al., 2007; Paoluzzi et al., 2008). In particular, MCL-1 has been reported to mediate TRAIL resistance in various cancer cells. Downregulation of MCL-1 by siRNA enhances TRAIL-induced apoptosis in cancers (Han et al., 2006; Meng et al., 2007; Wirth et al., 2005), and overexpression of mir-29b, which inhibits translation of MCL-1, makes cancer cells sensitive to TRAIL-induced apoptosis (Mott et al., 2007). MCL-1 expression is induced by TRAIL in TRAIL-resistant cells through an NF-κB-dependent pathway and disruption of TRAIL-induced MCL-1 sensitizes cells to apoptosis (Ricci et al., 2007). Consistent with previous studies, we have found

that TRAIL-induced MCL-1 is a critical factor for TRAIL resistance in human prostate cancer cells, not through NF-κB but rather through a p38 MAPK pathway. We did investigate the role of NF-κB in TRAIL resistance in DU145 cells and found that NF-κB was constitutively active. However, inhibition of NF-κB had no effect on TRAIL-induced cell death (data not shown). MCL-1 has also been shown to be regulated by several additional kinase-activated transcription factors (Epling-Burnette et al., 2001; Nijhawan et al., 2003; Puthier et al., 1999; Wang et al., 1999), and our results have shown that both p38 MAPK and JNK regulate the transcription of MCL-1, although it remains unclear which transcription factors are targeted. Nevertheless, we have shown for the first time that MCl-1 is transcriptionally regulated following TRAIL treatment, thereby leading to resistance.

Finally, there are several clinical implications that have arisen from our studies, particularly related to the use of small molecule BCL-2 antagonists and Smac mimetics. There is currently legitimate excitement surrounding the use of BCL-2 antagonists, such as ABT-737, in the treatment of tumors made resistant to conventional chemotherapy through overexpression of antiapoptotic BCL-2 family members. Similar to BAD, however, ABT-737 selectively targets BCL-2, BCL-XL, and BCL-W, but not MCL-1, and thus is unlikely to synergize with TRAIL in the treatment of MCL-1 overexpressing tumors. One potential approach for bypassing the antiapoptotic BCL-2 family members altogether could be to

utilize Smac mimetics, which antagonize IAPs and liberate active caspases-3 and -7 following their initial processing by caspase-8. Indeed, consistent with previous studies in other cancer cell types, from our laboratory and others, we found that release of Smac (rather than cytochrome c) was essential for TRAIL-induced apoptosis in DU145 prostate cancer cells (Fig. 3.7). Smac mimetics however reportedly induce apoptosis by stimulating autoubiquitination and degradation of cIAP1 and cIAP2, resulting in activation of the noncanonical NF-κB pathway and upregulation of TNF. Consequently, it remains unclear how effective these agents will be *in vivo* or whether they will exhibit significant side effects due to the production of TNF. Our studies suggest that TRAIL might instead be used effectively in combination with clinically available p38 MAPK inhibitors for the treatment of cancers expressing MCL-1.

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